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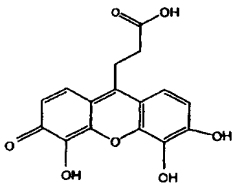
(54) Title: A METHOD OF INHIBITING VIRAL REPLICATION TARGETING THE NUCLEOCAPSID PROTEIN

Database structures and NC-p7 binding constants for structurally similar actives from primary screen.



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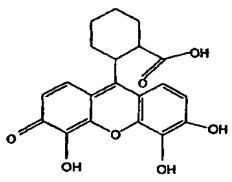

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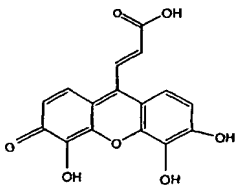

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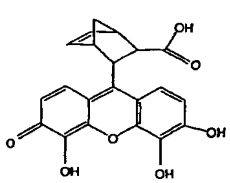

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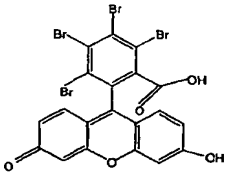

NSC 119889 (259nM)

(57) Abstract: The invention provides methods and pharmaceutical compositions for inhibiting viral replication, particularly retroviral replication. The methods comprise administration of tricyclic compounds that disrupt viral nucleocapsid binding to nucleic acids.

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CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional application 60/266,665, filed February 5, 2001, which is herein incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to anti-viral therapy and provides novel methods of inhibiting viral replication.

BACKGROUND OF THE INVENTION

It is well known that, under selection pressure, viruses often mutate to drug-resistant strains, thereby limiting the efficacy of most antiviral agents. Those viral structures that are required for viability and growth are often good drug targets because their inactivation cannot be easily overcome by mutation. The utility of these targets can be further enhanced if the structures are mutationally intolerant. Furthermore, these structures may be conserved and/or maintained between virus families, groups or genuses.

In particular retroviruses such as HIV, can become rapidly resistant to drugs used to treat the infection due to the high error rate of the reverse transcriptase enzyme responsible for transcribing its RNA genome. HIV is an example of such a hyper-mutable virus. It has diverged into two major species, HIV-1 and HIV-2, each of which has many clades, subtypes and drug resistant variations.

Strategies for coping with emergence of viral drug-resistant strains include combination drug therapies (Lange (1996) *AIDS* 10 Suppl 1:S27-S30). Drugs against different viral proteins and drugs against multiple sites on the same protein are commonly used as a strategy to overcome the adaptability of the virus. Combination therapies for retroviruses, using, *e.g.*, protease inhibitors and nucleoside analogues, such as AZT, ddI, ddC and d4T, can become ineffectual; the virus develops complete resistance in a relatively short period of time (Birch (1998) *AIDS* 12:680-681; Roberts (1998) *AIDS* 12:453-460; Yang (1997) *Leukemia* 11 Suppl 3:89-92; Demeter (1997) *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 14(2):136-144; Kuritzkes (1996) *AIDS* 10 Suppl 5:S27-S31). Furthermore, no

effective anti-retroviral vaccine is currently available (Bolognesi (1998) *Nature* 391:638-639; Bangham (1997) *Lancet* 350:1617-1621).

The HIV-1 caused AIDS epidemic began about 18 years ago. Since then the number of new cases have increased over time. By the end of 1994, 1,025,073 AIDS cases had been reported to the WHO, with a 20% increase in the number of cases since December, 1993 (Galli (1995) *Q. J. Nucl. Med.* 39:147-155). By the year 2000, the WHO predicts that there will be 30 to 40 million cumulative HIV-1 infections in the world (Stoneburner (1994) *Acta Paediatr. Suppl.* 400:1-4).

The Gag and Gag-Pol proteins in the *Retroviridae*, except for *Spumaviruses*, contain a highly conserved zinc finger motif (CCHC) within the nucleocapsid p7 (NCp7) protein portion of the polyprotein (see definitions, below). The absolute conservation of the metal chelating cysteine and histidine residues along with other residues of the protein and its in participation in essential functions during early and late virus replication has identified this feature as an antiviral target. Mutations of the chelating residues in the zinc fingers yield a non-infectious virus. Because zinc fingers are identical in most retroviruses, reagents able to inhibit its function have the potential of being broad spectrum anti-viral therapeutic drugs. For example, it has been shown that compounds that target the zinc finger by irreversible binding and cause ejection of the zinc molecule exhibit antiviral activity (*see, e.g.*, U.S. Patent 6,001,555; Rice *et al., Nature* 361:473-475, 1993). Disulfide benzamidines were also shown to be active in acutely and chronically infected cell lines (Rice *et al., Science* 270:1194-1197, 1995), and a series of pyridinioalkanoyl thioesters were developed that had superior anti-HIV-1 activity and less toxicity compared to the disulfide benzidines (*see, e.g.*, Turpin *et al., J. Med. Chem.* 42:67-86, 1999). A cyclic peptide that mimics several binding determinants in NC-p7 and inhibits NC-p7 annealing activities has also been designed (*e.g.*, Druillennec *et al., Proc Natl. Acad. Sci USA* 96:4886-4891, 1999; Druillennec *et al., Bioorg Med Chem Lett* 9:627-632, 1999).

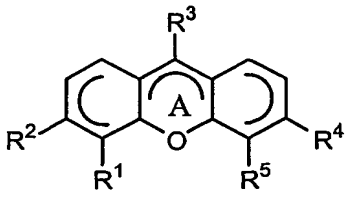
The present invention now provides new methods of inhibiting viral replication using tricyclic compounds that disrupt nucleocapsid/nucleic acid binding interactions.

SUMMARY OF THE INVENTION

The invention provides a method of inhibiting viral replication by administration of compounds that disrupt nucleocapsid binding to nucleic acids.

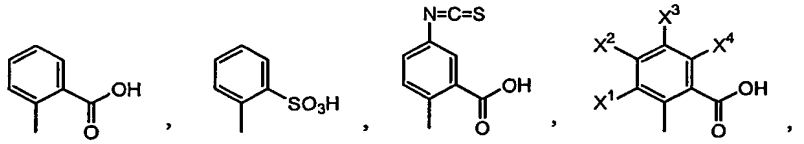
In one aspect, the invention provides a method of inhibiting replication of a virus, said method comprising:

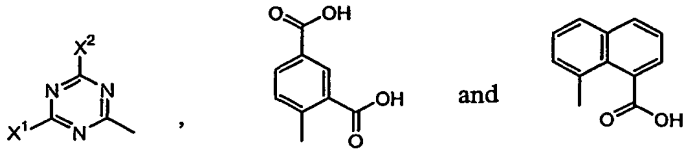
contacting a nucleocapsid protein of the virus with a compound having the formula:



wherein R^1 and R^5 are members independently selected from the group consisting of –H, –OH, and =O; R^2 and R^4 are members independently selected from the group consisting of H, -OH and =O; R^3 is a member selected from the group consisting of substituted or unsubstituted alkyl, substituted or unsubstituted alkylene, substituted or unsubstituted cycloalkyl and substituted or unsubstituted aryl; and ring system A is either saturated or mono-unsaturated. In another embodiment, R^1, R^5, R^2, and R^4 are –OH.

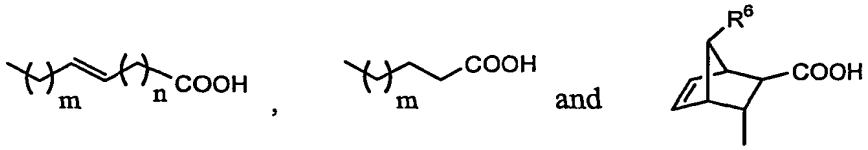
In one embodiment, R^3 is aryl substituted with one or more member selected from the group consisting of –COOH, $-SO_3H$, -N=C=S, halogen, and substituted or unsubstituted heteroaryl. Often, R^3 is a member selected from the group consisting of





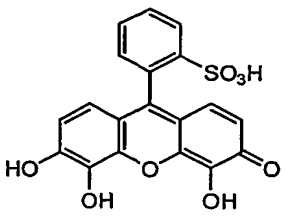
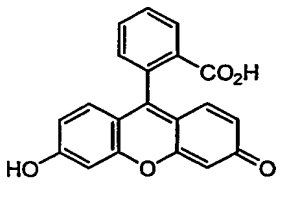
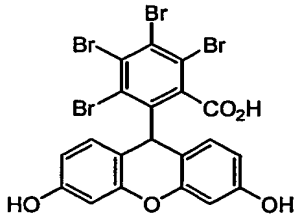
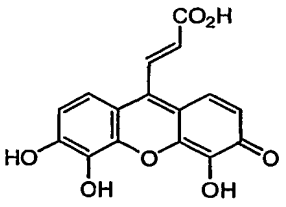
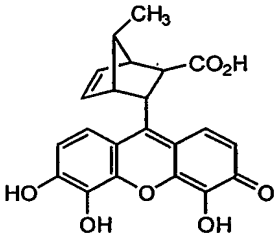
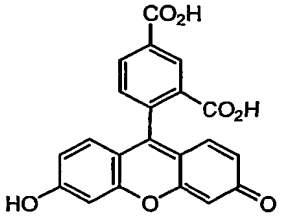
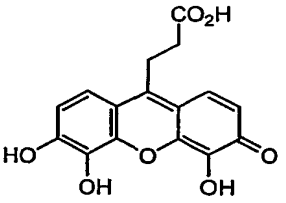
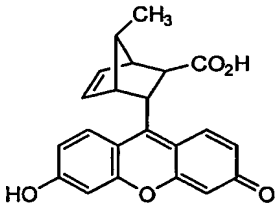
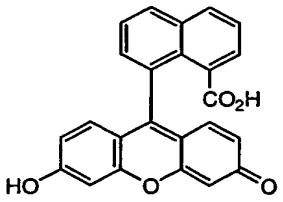
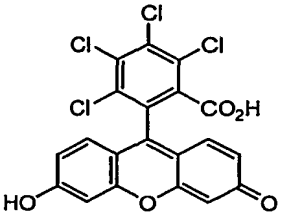
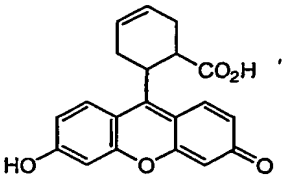
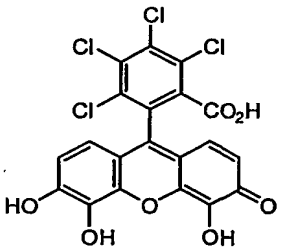
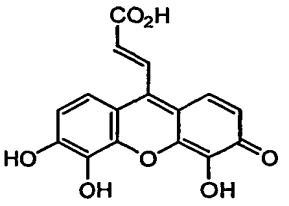
wherein X^1, X^2, X^3, and X^4 are members independently selected from the group consisting of -H, -F, -Cl, -Br, -I and –COOH.

In another embodiment, R^3 is a member selected from the group consisting alkyl substituted with –COOH, alkylene substituted with –COOH and cycloalkyl substituted with –COOH. Often R^3 is a member selected from the group consisting of



wherein R^6 is a member selected from substituted alkyl and unsubstituted alkyl; and m and n are independently integers from 0 to 10.

In exemplary embodiments, the compound is a member selected from the group consisting of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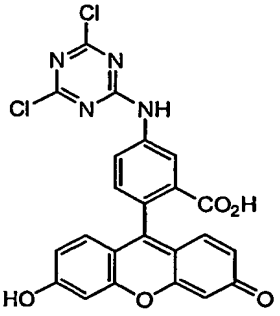
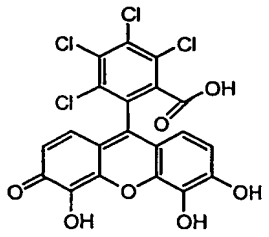


Other embodiments include:



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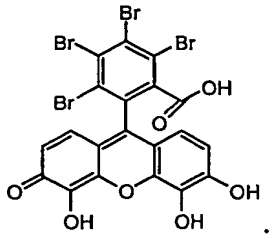


Particular embodiments of the invention include methods wherein the virus is a retrovirus. In some embodiments, the virus is a retrovirus derived from a avian sarcoma and leukosis retroviral group, a mammalian B-type retroviral group, a human T cell leukemia and bovine leukemia retroviral group, a D-type retroviral group, a murine leukemia-related group, or a lentivirus group. Often the retrovirus is an HIV-1, an HIV-2, an SIV, a BIV, an EIAV, a Visna, a CaEV, an HTLV-1, a BLV, an MPMV, an MMTV, an RSV, an MuLV, a FeLV, a BaEV, or an SSV retrovirus. Preferably, the retrovirus is HIV-1.

In the method for inactivating a virus, the contacting of the virus with the compound can be performed *in vivo*. In this embodiment, the compound can be administered to inhibit the transmission of the virus. The compound can be administered intra-vaginally or intra-rectally to inhibit the transmission of the virus. The compound can be administered to a human as a pharmaceutical formulation. The compound can be administered to an animal as a veterinary pharmaceutical formulation. The method further comprises contacting the virus with a non-tricyclic anti-retroviral agent. The anti-retroviral agent can be a nucleoside analogue, a protease inhibitor, or a non-nucleoside reverse trancriptase inhibitor (NNRTI). The nucleoside analogue can be AZT, ddCTP or DDI. The protease inhibitor can be Indinavir, Saquinavir, or Ritonavir and NNRTI include nevirapine and efavirenz.

In another aspect, the invention also provides a method for inactivating a virus, wherein the contacting of the virus with the compound can be performed *in vitro*. In this embodiment of the method, the contacting of the retrovirus with the compound can be performed in a blood product, blood plasma, nutrient media, protein, a pharmaceutical, a cosmetic, a sperm or oocyte preparation, cells, cell cultures, bacteria, viruses, food or drink.

In one aspect of the methods of the invention, the compound is administered to a human as a pharmaceutical formulation. Often the compound is administered intra-vaginally or intra-rectally to inhibit the transmission of the virus.

In another embodiment, the compound is administered to an animal as a veterinary pharmaceutical formulation. Preferably, the pharmaceutical formulation

comprises a unit dose of a tricyclic compound described herein. Often, the pharamceutical formulation further comprises a pharmaceutical exceipient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the names and numbering system for gallein and lfuorescein derivative test compounds described in the Example section. The numbering system for the benzoic acid group will change, depending on the form of the molecule (lactoid or quinoid) and the substituents on the ring on the benzoic acid ring system.

Figure 2 shows database structures and NC-p7 binding constants for structurally similar actives from primary screen. Structures of actives as listed in the NCI chemistry database. Dissociation constants were determined using solution inhibition analysis on the BIAcore system.

Figure 3 shows the correlation between Kd for compound binding to NC-p7 by solution inhibition or quenching of tryptophan fluorescnece. Dissociation constants were calculated using either solution inhibition or tryptophan fluorescence quenching methods. The data were fit using linear regression (solid line, r2=0.97), confidence levels were set at 99% (dotted lines).

Figure 4 shows the results of a solution inhibition of NC-p7 with 157412-10 and fluorescein. NC-p7 (50nM) was incubated with an increasing concentration of either 157512-10 or fluorescein. Uninhibited NC-p7 bound to d(TG)4 immobilized on a BIAcore chip, the amount of NC-p7 was calculated using a standard curve.

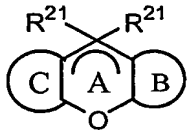
Figure 5 shows the binding of 157412-10 to NC-p7 mutants. The direct binding of 157412-10 to NC-p7 was monitored by the quenching of the tryptophan fluorescence (excitation 295nm, emission 350nm). NC-p7 wild type and mutants (400nM) were incubated with increasing concentrations of 157412-10.

Figures 6a and 6b. Structures of synthetic variants of NSC 157412-10 are shown in Figure 6a. Figure 6b shows the solution inhibition of NC-p7 with synthetic variants of NSC 157412-10.

Figure 7 shows the antiviral activity of 157412-10 and related compounds. Figures 7A and 7B shows the effects of NSC 157412-10 and its synthetic variants on early stages of HIV replication assessed in MAGI cells. (A) MAGI cells were infected with HIVIIIB and cultured in the absence or presence of increasing concentrations of NSC 157412-10 or its synthetic version, 4436-42-1. The anti-HIV activity is expressed as the percent HIV infected cells of untreated control. Both compounds were tested in triplicate at

each dose. The values shown are mean with SD. NSC 157412-10 and 4436-42-1 exhibited similar levels of anti-HIV activity in a dose dependent manner. (B) MAGI cells were infected with HIVIIIB or HIVNL4-3 and cultured in the absence or presence of increasing concentrations of 4436-42-1, 4436-26-1, or 4436-8-1. All compounds were tested in triplicate at each dose. The degree of HIV inhibition was expressed as the percent reduction in HIV infected cells compared to untreated control. The values shown are mean with SD. The 4436-42-1 and 4436-26-1 consistently exhibited a potent anti-HIV activity, while 4436-8-1 had only limited activity. The experiment shown is representative of 3 separate experiments.

Figure 8 depicts exemplary embodiments of compounds having a structure according to Formula II:



(II)

Structures of the active compounds are as listed in the NCI chemistry database. Dissociation constants were determined using solution inhibition analysis on the BIAcore system.

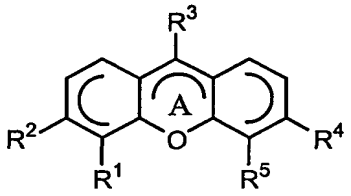
DETAILED DESCRIPTION OF THE INVENTION

The efficacy of most antiviral agents is limited because it is common that, under selection pressure, viruses mutate to drug-resistant strains. Development of drug resistance is a survival strategy particularly pronounced amongst retroviruses because of their ability to rapidly mutate. Viral structures that are required for viability and replication are typically considered as good drug targets because their inactivation cannot be easily overcome by mutation, thus often these structure are mutationally intolerant. Furthermore, these structures maybe conserved and/or maintained between virus families, groups or genuses, thus providing a common target for the development of antiviral agents or therapies.

HIV-1's nucleocapsid (NC) protein, NCp7, contains two zinc fingers separated by only seven amino acids (Henderson (1992) *J. Virol.* 66:1856). Both fingers are essential for infectivity (Aldovini (1990) *J. Virol.* 64:1920; Gorelick (1990) *J. Virol.* 64:3207). Agents have been identified that target this regions, *see, e.g.*, WO97/44064, WO9965871, U.S. Patents 6,001,555 and 6,046,228. The present invention employs compounds comprising a

tricyclic ring structure which bind to the nucleocapsid with high affinity and thereby inhibit interaction with nucleic acid molelcules.

In a first aspect of the invention, there is provided a method of inhibiting viral replication using compounds having a structure according to Formula I:

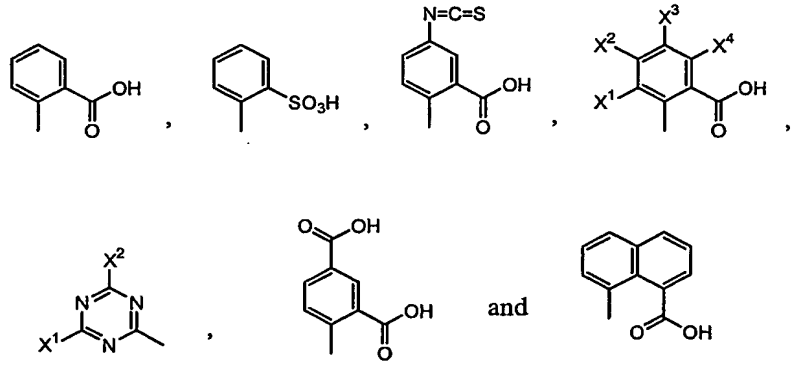


(I)

In Formula I, R^1 and R^5 are members independently selected from –H, –OH, and =O. R^2 and R^4 are members independently selected from H, -OH and =O. R^3 is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted alkylene, substituted or unsubstituted cycloalkyl and substituted or unsubstituted aryl. Ring system A is fully saturated or mono-unsaturated.

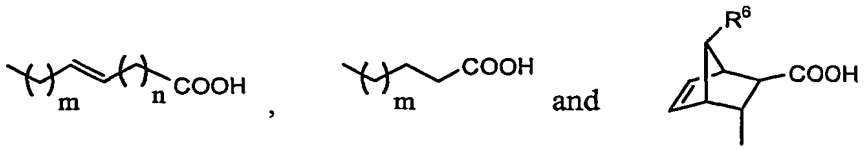
In a preferred embodiment, the method of the invention utilizes compounds in which R^3 is aryl substituted with one or more member selected from the group consisting of -COOH, -SO_3H, -N=C=S, halogen, and substituted or unsubstituted heteroaryl.

In yet another preferred embodiment, the method makes use of compounds in which R^3 is a member selected from the group consisting of:



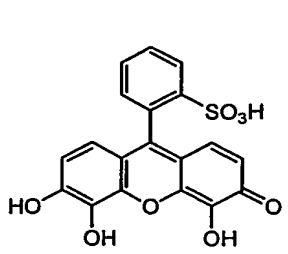
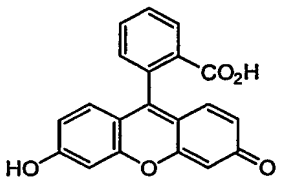
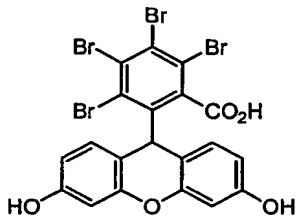
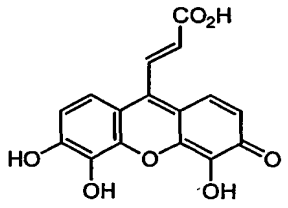
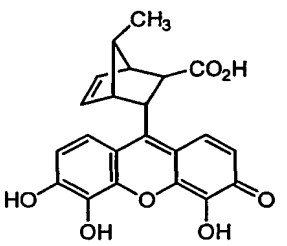
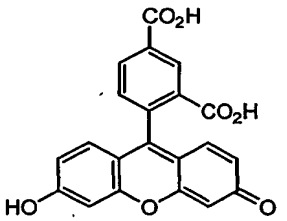
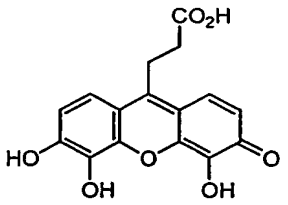
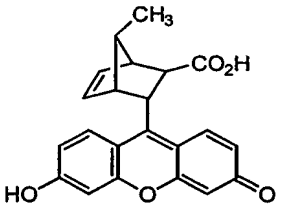
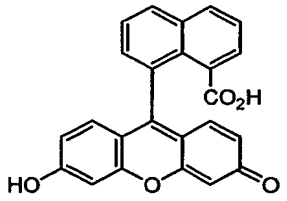
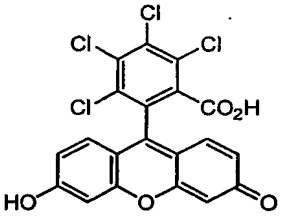
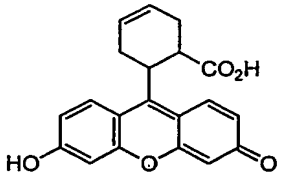
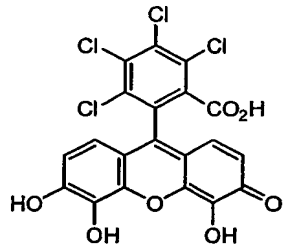
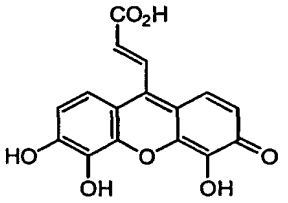
wherein X^1, X^2, X^3, and X^4 are members independently selected from the group consisting of -H, -F, -Cl, -Br, -I and –COOH.

In a still further preferred embodiment, the method of the invention utilizes compounds in which R^3 is a member selected from the group consisting alkyl substituted with –COOH, alkylene substituted with –COOH and cycloalkyl substituted with –COOH. R^3 is even more preferably, a member selected from:



wherein R^6 is a member selected from substituted alkyl and unsubstituted alkyl; and m and n are independently integers from 0 to 10.

Still further preferred embodiments of the method utilize compounds having one or more structures selected from the group consisting of: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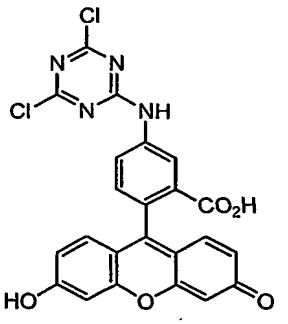
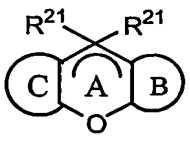


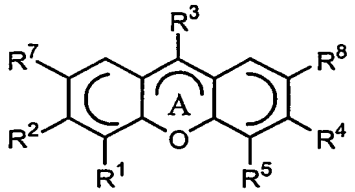
In other embodiments of the invention, the methods rely on the use of compounds having a structure according to Formula II:



(II)

wherein ring systems C and B are members independently selected from substituted or unsubstituted cycloalkyl and substituted or unsubstituted aryl groups. The symbols R^{21} and R^{22} represent groups that are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, and substituted or unsubstituted heteroaryl. Ring system A is either fully saturated or mono-unsaturated. Presently preferred compounds of use in the present invention having the structural motif set forth in Formula II are displayed in FIG. 1.

In another embodiment, the method of the invention utilizes compounds having a structure according to Formula III:



(III)

In Formula III, R^1 and R^5 are members independently selected from the group consisting of –H, –OH, and =O. R^2 and R^4 are members independently selected from H, -OH and =O. R^7 and R^8 are members independently selected from $–NO_2$, halo, -OH, and substituted or unsubstituted alkyl. R^3 is a member selected from the group consisting of substituted or unsubstituted alkyl, substituted or unsubstituted alkylene, substituted or unsubstituted cycloalkyl and substituted or unsubstituted aryl; and ring system A is fully saturated or mono-unsaturated.

The present invention is directed to the use of tricyclic compounds described herein to disrupt the association of a viral nucleocapsid protein to nucleic acid. Many of these compounds are known, see, *e.g.*, the National Cancer Institute chemical repository although none have been used as anticancer (or other indication) agents prior to the present application. Chemical structural information on some of the claimed compound is available via the DTP website: http://dtp.nci.nih.gov. Methods of synthesizing such chemicals are known to those of skill in the art. Moreover, new tricyclic compounds according to Formula I, II, or III that inhibit viral replication as described herein can be synthesized using techniques readily apparent to those of skill in the art. Examples of the synthesis of tricyclic compounds in accordance with Formula I, II, and III can be found in U.S. Patent 5,637,733; Sun, *et al.*, *J. Org. Chem.* 1997, 62:6469-6475; U.S. Patent 5,395,862 and EP515133.

Definitions

To facilitate understanding the invention, a number of terms are defined below.

A "tricyclic compound" as used herein refers to a compound having a structure corresponding to that set out in Formula I, II, or III. These formulas define a three-dimensional phamacophore which interacts with the nucleocapsid protein by forming at least one hydrogen bond. Other tricyclic compounds to exploit the present and other hydrogen bonding modalities will be apparent to those of skill in the art.

The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups which are limited to hydrocarbon groups are termed "homoalkyl".

The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $-CH_2CH_2CH_2CH_2-$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

The terms "alkoxy," "alkylamino" and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, or a sulfur atom, respectively.

The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and from at least one

heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-CH_2-O-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-CH_2-CH_2,-S(O)-CH_3$, $-CH_2-CH_2-S(O)_2-CH_3$, $-CH=CH-O-CH_3$, $-Si(CH_3)_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. Up to two heteroatoms may be consecutive, such as, for example, $-CH_2-NH-OCH_3$ and $-CH_2-O-Si(CH_3)_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $-CH_2-CH_2-S-CH_2CH_2-$ and $-CH_2-S-CH_2-CH_2-NH-CH_2-$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (*e.g.*, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2R-$.

The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of "alkyl" and "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of heterocycloalkyl include, but are not limited to, 1 -(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1 -piperazinyl, 2-piperazinyl, and the like.

The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C_1-C_4)alkyl" is mean to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

The term "aryl" means, unless otherwise stated, a polyunsaturated, aromatic, hydrocarbon substituent which can be a single ring or multiple rings (up to three rings) which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from zero to four heteroatoms selected from N, O, and S, wherein the nitrogen

and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below.

For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

Each of the above terms (*e.g.*, "alkyl," "heteroalkyl," "aryl" and "heteroaryl") are meant to include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

Substituents for the alkyl and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', -CO_2R', -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', -S(O)R', -$S(O)_2$R', -$S(O)_2$NR'R", -$NRSO_2$R', -CN and -NO_2 in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R", R"' and R"" each preferably independently refer to hydrogen, and heteroalkyl, unsubstituted aryl, aryl substituted with 1-3 halogens, unsubstituted alkyl, alkoxy or thioalkoxy groups, or aryl-(C_1-C_4)alkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-

membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, $-CF_3$ and $-CH_2CF_3$) and acyl (*e.g.*, $-C(O)CH_3$, $-C(O)CF_3$, $-C(O)CH_2OCH_3$, and the like).

Similar to the substituents described for the alkyl radical, substituents for the aryl and heteroaryl groups are varied and are selected from, for example: halogen, -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', $-CO_2R'$, -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', $-NR"C(O)_2R'$, -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', -S(O)R', $-S(O)_2R'$, $-S(O)_2NR'R"$, $-NRSO_2R'$, -CN and $-NO_2$, -R', $-N_3$, $-CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R", R"' and R"" are preferably independently selected from hydrogen, (C_1-C_8)alkyl and heteroalkyl, unsubstituted aryl and heteroaryl, (unsubstituted aryl)-(C_1-C_4)alkyl, and (unsubstituted aryl)oxy-(C_1-C_4)alkyl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present.

Two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-T-C(O)-(CRR'_2)_q-U-$, wherein T and U are independently -NR-, -O-, -CRR'- or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-A-(CH_2)_r-B-$, wherein A and B are independently –CRR'-, -O-, -NR-, -S-, -S(O)-, $-S(O)_2-$, $-S(O)_2NR'-$ or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula $-(CRR')_s-X-(CR"R"')_t-$, where s and t are independently integers of from 0 to 3, and X is -O-, -NR'-, -S-, -S(O)-, $-S(O)_2-$, or $-S(O)_2NR'-$. The substituents R, R', R" and R"' are preferably independently selected from hydrogen or substituted or unsubstituted (C_1-C_6)alkyl.

As used herein, the term "heteroatom" is meant to include oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

The term "pharmaceutically acceptable salts" is meant to include salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of

the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium, potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (*see*, for example, Berge *et al.*, "Pharmaceutical Salts", *Journal of Pharmaceutical Science*, 1977, *66*, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

The neutral forms of the compounds are preferably regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

In addition to salt forms, the present invention provides compounds, which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example, prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

Certain compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are encompassed within the scope of the present

invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are encompassed within the scope of the present invention.

"Contacting" refers to the act of bringing components of a reaction into adequate proximity such that the reaction can occur. More particularly, as used herein, the term "contacting" can be used interchangeably with the following: combined with, added to, mixed with, passed over, flowed over, etc.

As used herein, the term "Gag-Pol protein" refers to the polyprotein translation product of HIV-1 or other retroviruses, as described, *e.g.*, by Fehrmann (1997) *Virology* 235:352359; Jacks (1988) *Nature* 331:280-283. The "Gag protein" is processed by a viral protease to yield mature viral proteins, *see, e.g.*, Humphrey (1997) *Antimicrob. Agents Chemother.* 41:1017-1023; Karacostas (1993) *Virology* 193:661-671.

The term "halogen" is used herein to refer to fluorine, bromine, chlorine and iodine atoms.

As used herein, "isolated," when referring to a molecule or composition, such as, for example, a tricyclic compound of the invention, a tricyclic-complexed polypeptide or virus, or a tricyclic-inactivated virus, means that the molecule or composition is separated from at least one other compound, such as a protein, other nucleic acids (*e.g.*, RNAs), or other contaminants with which it is associated *in vivo* or in its naturally occurring state. Thus, a compound, polypeptide or virion is considered isolated when it has been isolated from any other component with which it is naturally associated, *e.g.*, cell membrane, as in a cell extract, serum, and the like. An isolated composition can, however, also be substantially pure. An isolated composition can be in a homogeneous state and can be in a dry or an aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemistry techniques such as polyacrylamide gel electrophoresis (SDS-PAGE) or high performance liquid chromatography (HPLC).

As used herein, the term "nucleocapsid protein" or "NC protein" refers to the retroviral nucleocapsid protein, which is an integral part of the virion nucleocapsid, where it coats the dimeric RNA genome, as described by, *e.g.*, Huang (1997) *J. Virol.* 71:4378-4384; Lapadat-Tapolsky (1997) *J. Mol. Biol.* 268:250-260. HIV-1's nucleocapsid protein is termed "NCp7," see also Demene (1994) *Biochemistry* 33:11707-11716.

All NC proteins of the *Oncovirinae* and *Lentivirinae* subfamilies of *Retroviridae* contain sequences of 14 amino acids with 4 invariant residues, $Cys(X)_2Cys(X)_4His(X)_4Cys$, (L.E. Henderson *et al. J. Biol. Chem.* 256, 8400 (1981)) which chelate zinc through histidine imidazole and cysteine thiolates with a K_d less than 10^{-13} (J.M. Berg, *Science* 232, 485 (1986); J.W. Bess, Jr., *et al., J. Virol.* 66, 840 (1992); M.R. Chance *et al., Proc. Natl. Acad. Sci. U.S.A.* 89, 10041 (1992); T.L. South and M.F. Summers, *Adv. Inorg. Biochem.* 8, 199 (1990); T.L. South, *et al., Biochem. Pharmacol.* 40, 123 (1990)). These structures are referred to as retroviral CCHC zinc fingers, and are one of the most highly conserved features of retroviruses. Examples of retroviruses which possess at least one CCHC type zinc finger per nucleocapsid protein include, but are not limited to, HIV-1, HIV-2, SIV, BIV, EIAV, Visna, CaEV, HTLV-1, BLV, MPMV, MMTV, RSV, MuLV, FeLV, BaEV, and SSV.

The term "retrovirus" as used herein refers to viruses of the *Retroviridae* family, which typically have ssRNA transcribed by reverse transcriptase, as defined by, *e.g.*, P. K. Vogt, "Historical introduction to the general properties of retroviruses," in Retroviruses, eds. J. M. Coffin, S. H. Hughes and H. E. Varmus, Cold Spring Harbor Laboratory Press, 1997, pp 1-26; Murphy *et al.* (eds.) Archives of Virology/Supplement 10, 586 pp (1995) Springer Verlag, Wien, NY; and the web site for the Committee on International Taxonomy of Viruses, Virology Division of the International Union of Microbiology Society at http://www.ncbi.nlm.nih.gov/ ICTV/ for viral classification and taxonomy. *Retroviridae* family members containing zinc finger motif-containing polypeptides and whose replication can be inhibited by the tricyclic compounds of the invention include avian sarcoma and leukosis retroviruses (alpharetroviruses), mammalian B-type retroviruses (betaretrovirus) (*e.g.*, mouse mammary tumor virus), human T cell leukemia and bovine leukemia retroviruses (deltaretroviruses) (*e.g.*, human T-lymphotropic virus 1), murine leukemia-related group (gammaretroviruses), D-type retroviruses (epsilonretrovirus) (*e.g.*, Mason-Pfizer monkey virus), and *Lentiviruses*. *Lentiviruses* include bovine, equine, feline, ovine/caprine, and primate lentivirus groups, such as human immunodeficiency virus 1 (HIV-1). Examples of particular species of viruses whose replicative capacity is affected by the tricyclic compounds of the invention include HIV-1, HIV-2, SIV, BIV, EIAV, Visna, CaEV, HTLV-1, BLV, MPMV, MMTV, RSV, MuLV, FeLV, BaEV, and SSV retrovirus.

Other viruses that include proteins with zinc finger domains that interact with nucleic acid can also be targeted with the tricyclic compounds as described herein. For example, human papilloma virus E6 and E7 proteins contain zinc finger domains that can be

targets for binding by this series of compounds (Beerheide *et al.*, *J Natl Cancer Inst* 91:1211-20, 1999). Likewise, the hepatitis C virus genome codes for zinc finger-containing protens that can be targeted with the tricyclic compounds.

Determining Anti-Viral Activity of the Tricyclic Compounds of the Invention

In determining the anti-viral activity of a tricyclic compound of the invention, the ability of the compound to bind to a viral nucleocapsid protein is often evaluated. The assessment of binding affinity can be determined using techniques known by one or ordinary skill in the art (see, e.g., WO97/44064). For example tryptophan fluorescence quenching or other binding assays such as the BIAcore chip technology can be used in order to determine the binding affinity of a tricyclic compound for the viral zinc finger-containing protein. Examples of these procedures are further provided in Example 1. As appreciated by one of skill, other assay procedures can be used to provide an equivalent assessment for binding of compounds to any viral nucleocapsis protein. In some embodiments, binding affinity is assessed and compounds that bind to the nucleocapsid at a K_D of less than 200 μM, determined, *e.g.*, using a solution inhibition assay as described in the Examples, are then analyzed for anti-viral activity *in vitro*.

A tricyclic compound is within the scope of the invention if it displays any antiviral activity (*i.e.*, any ability to decrease the cytopathic effect or diminish the transmission of or the replicative capacity of a virus). The antiviral activity can be determined empirically by clinical observation or objectively using any *in vivo* or *in vitro* test or assay, *e.g.*, the XTT cytoprotection assay (described herein), measuring Tat-induced activity (as in the HeLa-CD4-LTR-beta-gal (MAGI cells) assay and detecting Tat-induced beta-galactosidase activity, *see, e.g.*, Tokunaga (1998) *J. Virol.* 72:6257-6259), and the like. A tricyclic compound with any degree of measurable antiviral activity is within the scope of the invention.

One exemplary means to determine antiviral activity is with CEM-SS cells and virus (*e.g.*, HIV-1_{RF}) (MOI = 0.01) using the XTT (2,3-bis[2-methoxy- 4-nitro-5-sulfophenyl] -5- [(phenylamino)carbonyl]-2H-tetrazolium hydroxide) cytoprotection assay (*see, e.g.*, Weislow, et al, *J. Natl. Canc. Inst.* 81: 577-586, 1989; Rice *PNAS* 90:9721-9724, 1993; and Rice *Antimicrob. Agents Chemother.* 41:419-426, 1997). Briefly, cells are infected with HIV-1_{RF} (or other virus to be tested) in the presence of various dilutions of test compounds (tricyclic compounds and controls). The cultures are incubated for seven days. During this time control cultures without protective compounds (*i.e.*, compounds with anti-

viral activity) replicate virus, induce syncytia, and result in about 90% cell death. The cell death is measured by XTT dye reduction. XTT is a soluble tetrazolium dye that measures mitochondrial energy output, similar to MTT. Positive controls using dextran sulfate (an attachment inhibitor) or 3'-Azido -2'-3'-dideoxythymidine, AZT (a reverse transcriptase inhibitor) are added to each assay. Individual assays are done in duplicate using a sister plate method.

Effective antiviral concentrations providing 50% cytoprotection (EC_{50}), and cellular growth inhibitory concentrations causing 50% cytotoxicity (IC_{50}) are calculated.

Alternatively, any virus can be grown in culture, or in an *in vivo* (animal) model, in the presence or absence of a tricyclic compound or a tricyclic-containing pharmaceutical formulation to test for anti-viral, viral transmission-inhibiting activity and efficacy. Any virus, assay or animal model which would be apparent to one of skill upon review of this disclosure can be used, *see, e.g.*, Lu (1997) *Crit. Rev. Oncog.* 8:273-291; Neildez (1998) Virology 243:12-20; Geretti (1998) *J. Gen. Virol.* 79:415-421; Mohri (1998) *Science* 279:1223-1227; Lee (1998) *Proc. Natl. Acad. Sci. USA* 95:939-944; Schwiebert (1998) *AIDS Res. Hum. Retroviruses* 14:269-274.

For *in vitro* assays, any measurable decrease in the viral load of a culture grown in the presence of a tricyclic test compound as compared to a positive or negative control compound is indicative of an anti-viral, transmission-inhibiting effect. Typically, at least a 30% reduction in viral load observed, generally, between 10% and 99%. As discussed in definition section, above, any relevant criteria can be used to evaluate the antiviral efficacy of a tricyclic composition or tricyclic-containing formulation.

Cloning and Expression of Viral Nucleocapsid Proteins

The tricyclic compounds of the invention of the invention prevent the binding of viral nucleocapsid protein to nucleic acids. In order to identify such compounds, the ability of tricyclic compounds to bind to the viral nucleocapsid is assessed in a binding assay using the targeted nucleocapsid protein, often a retroviral protein. The viral nucleocapsid proteins to detect the binding and antiviral activity of the tricyclic compounds are typically produced using recombinant technology. General laboratory procedures for the cloning and expression of nucleocapsid proteins can be found, *e.g.*, current editions of Sambrook, *et al.*, Molecular Cloning *A Laboratory Manual* (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989; CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. Greene Publishing and Wiley-Interscience, New

York (1987). Sequences and sources of nucleocapsid proteins, including nucleic acids, proteins and viral sources, are publicly available, for example, through electronic databases, such as, *e.g.*, The National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/Entrez/, or, The National Library of Medicine at http://www.ncbi.nlm.nih.gov/PubMed/.

In general, the DNA encoding the polypeptide or peptide of interest are first cloned or isolated in a form suitable for ligation into an expression vector. After ligation, the vectors containing the DNA fragments or inserts are introduced into a suitable host cell for expression of the recombinant polypeptides. The polypeptides are then isolated from the host cells. The nucleic acids may be present in transformed or transfected whole cells, in a transformed or transfected cell lysate, or in a partially purified or substantially pure form. Techniques for nucleic acid manipulation of genes encoding zinc finger-containing proteins, such as subcloning nucleic acid sequences into expression vectors, labeling probes, DNA hybridization, and the like are described, *e.g.*, in Sambrook and Ausubel, *supra.*

Once the DNAs are isolated and cloned, the desired polypeptides are expressed in a recombinantly engineered cell such as bacteria, yeast, insect, or mammalian cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of the recombinantly produced proteins. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made. In brief summary, the expression of natural or synthetic nucleic acids encoding polypeptides will typically be achieved by operably linking the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding recombinant polypeptides. To obtain high level expression of a cloned gene, it is desirable to construct expression plasmids which contain, at the minimum, a promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator.

Viricidal Activity of Tricyclic Compounds of the Invention

In another aspect, the invention also provides a method of using a composition comprising a bio-organic or other material and an amount of a tricyclic compound of the

invention effective to inactivate any virus (susceptible to inactivation by a tricyclic compound) which is or may contaminate the material. The material can be bio-organic, such as, *e.g.*, blood plasma, nutrient media, protein, a pharmaceutical, a cosmetic, a sperm or oocyte preparation, cells, cell cultures, bacteria, viruses, foods, drinks. They can be surgical or other medical materials, such as, *e.g.*, implant materials or implantable devices (*e.g.*, plastics, artificial heart valves or joints, collagens), medical materials (*e.g.*, tubing for catheterization, intubation, IVs) and containers (*e.g.*, blood bags, storage containers), and the like. Alternatively, a tricyclic compound of the invention can be in the form of a composition which is applied to any of the above materials as a viricidal reagent and removed before the material's use. The viricidal composition can contain a mixture of different tricyclic compounds of the invention in varying amounts. For example, tricyclic compounds can be added to cell cultures to reduce the likelihood of viral contamination, providing added safety for the laboratory workers.

Tricyclic Compounds as Pharmaceutical Formulations

The invention also provides pharmaceutical formulations comprising the tricyclic compounds of the invention. These tricyclic compounds are used in pharmaceutical compositions that are useful for administration to mammals, particularly humans, for the treatment of viral, especially retroviral, infections.

The compounds of the invention can be formulated as pharmaceuticals for administration in a variety of ways. Typical routes of administration include both enteral and parenteral. These include, *e.g.*, without limitation, subcutaneous, intramuscular, intravenous, intraperitoneal, intramedullary, intrapericardiac, intrabursal, oral, sublingual, ocular, nasal, topical, transdermal, transmucosal, or rectal. The mode of administration can be, *e.g.*, via swallowing, inhalation, injection or topical application to a surface (*e.g.*, eyes, mucous membrane, skin). Particular formulations typically are appropriate for specific modes of administration. Various contemplated formulations include, *e.g.*, aqueous solution, solid, aerosol, liposomal and transdermal formulations. Details on techniques for formulation and administration are well described in the scientific and patent literature, *see, e.g.*, the latest edition of "Remington's Pharmaceutical Sciences" (Maack Publishing Co, Easton PA).

Aqueous Solutions for Enteral, Parenteral Or Transmucosal Administration

Examples of aqueous solutions that can be used in formulations for enteral, parenteral or transmucosal drug delivery include, *e.g.*, water, saline, phosphate buffered

saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions and the like. The formulations can contain pharmaceutically acceptable auxiliary substances to enhance stability, deliverability or solubility, such as buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. Additives can also include additional active ingredients such as bactericidal agents, or stabilizers. For example, the solution can contain sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate or triethanolamine oleate. These compositions can be sterilized by conventional, well-known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

Aqueous solutions are appropriate for injection and, in particular, for intravenous injection. The intravenous solution can include detergents and emulsifiers such as lipids. Aqueous solutions also are useful for enteral administration as tonics and administration to mucous or other membranes as, *e.g.*, nose or eye drops. The composition can contain a tricyclic compound in an amount of about 1 mg/ml to 100 mg/ml, more preferably about 10 mg/ml to about 50 mg/ml.

Formulations For Enteral Or Transdermal Delivery

Solid formulations can be used for enteral administration. They can be formulated as, *e.g.*, pills, tablets, powders or capsules. For solid compositions, conventional nontoxic solid carriers can be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10%-95% of active ingredient.

A non-solid formulation can also be used for enteral (oral) administration. The carrier can be selected from various oils including those of petroleum, animal, vegetable or synthetic origin, *e.g.*, peanut oil, soybean oil, mineral oil, sesame oil, and the like. See Sanchez, *et al.*, U.S. Patent No. 5,494,936. Suitable pharmaceutical excipients include starch, cellulose, talc, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene glycol, water, ethanol, and the like. Nonionic block copolymers synthesized from ethylene oxide and propylene oxide can also be pharmaceutical excipients;

copolymers of this type can act as emulsifying, wetting, thickening, stabilizing, and dispersing agents, *see, e.g.*, Newman (1998) *Crit. Rev. Ther. Drug Carrier Syst.* 15:89-142.

A unit dose form, such as a tablet, can be between about 50 mg/unit to about 2 grams/unit, preferably between about 100 mg/unit to about 1 gram/unit.

Topical Administration: Transdermal/ Transmucosal Delivery

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated can be used in the formulation. Such penetrants are generally known in the art, and include, *e.g.*, for transmucosal administration, bile salts and fusidic acid derivatives. In addition, detergents can be used to facilitate permeation. Transmucosal administration can be through nasal sprays, for example, or using suppositories.

For topical administration, the agents are formulated into ointments, creams, salves, powders and gels. In one embodiment, the transdermal delivery agent can be DMSO. Transdermal delivery systems can also include, *e.g.*, patches.

The tricyclic compounds can also be administered in sustained delivery or sustained release mechanisms, which can deliver the formulation internally. For example, biodegradeable microspheres or capsules or other biodegradeable polymer configurations capable of sustained delivery of a composition can be included in the formulations of the invention (*see, e.g.*, Putney (1998) *Nat. Biotechnol.* 16:153-157).

Formulation Delivery By Inhalation

For inhalation, the tricyclic compound formulation can be delivered using any system known in the art, including dry powder aerosols, liquids delivery systems, air jet nebulizers, propellant systems, and the like. *See, e.g.*, Patton (1998) *Biotechniques* 16:141-143; inhalation delivery systems by, *e.g.*, Dura Pharmaceuticals (San Diego, CA) , Aradigm (Hayward, CA), Aerogen (Santa Clara, CA), Inhale Therapeutic Systems (San Carlos, CA), and the like.

For example, the pharmaceutical formulation can be administered in the form of an aerosol or mist. For aerosol administration, the formulation can be supplied in finely divided form along with a surfactant and propellant. The surfactant preferably is soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic

anhydride such as, for example, ethylene glycol, glycerol, erythritol, arabitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or natural glycerides can be employed. The surfactant can constitute 0.1%-20% by weight of the composition, preferably 0.25%-5%. The balance of the formulation is ordinarily propellant. Liquefied propellants are typically gases at ambient conditions, and are condensed under pressure. Among suitable liquefied propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above can also be employed. In producing the aerosol, a container equipped with a suitable valve is filled with the appropriate propellant, containing the finely divided compounds and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve. *See, e.g.*, Edwards (1997) *Science* 276:1868-1871.

A nebulizer or aerosolizer device for administering tricyclic compounds of this invention typically delivers an inhaled dose of about 1 mg/m^3 to about 50 mg/m^3.

Delivery by inhalation is particular effective for delivery to respiratory tissues for the treatment of respiratory conditions including an inflammatory component.

Other Formulations

In preparing pharmaceuticals of the present invention, a variety of formulation modifications can be used and manipulated to alter pharmacokinetics and biodistribution. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art. For a general discussion of pharmacokinetics, *See, Remington's Pharmaceutical Sciences, supra*, Chapters 37-39.

Administration

The tricyclic compound of the invention are used in the treatment and prevention of viral infection, particularly, retroviral infections. The amount of tricyclic compound adequate to accomplish this is defined as a "therapeutically effective dose." The dosage schedule and amounts effective for this use, *i.e.*, the "dosing regimen," will depend upon a variety of factors, including frequency of dosing, the stage of the disease or condition, the severity of the disease or condition, the general state of the patient's health, the patient's physical status, age and the like. In calculating the dosage regimen for a patient, the mode of administration also is taken into consideration.

The dosage regimen must also take into consideration the pharmacokinetics, *i.e.*, the tricyclic compound's rate of absorption, bioavailability, metabolism, clearance, and the like (*see, e.g.*; the latest Remington's edition, *supra*).

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should pro-vide a quantity of a tricyclic compound sufficient to treat the patient effectively. The total effective amount of a tricyclic compound of the present invention can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which the multiple doses are administered over a more prolonged period of time. One skilled in the art would know that the concentration of a tricyclic compound of the present invention required to obtain an effective dose in a subject depends on many factors including, *e.g.*, the pharmacokinetics of the prodrug and of its hydrolysis product, the age and general health of the subject, the route of administration, the number of treatments to be administered and the judgment of the prescribing physician. In view of these factors, the skilled artisan would adjust the dose so as to provide an effective dose for a particular use.

As appreciated by one of skill in the art, the tricyclic compounds can be used in conjunction with other therapies used for the treatment of viral infection. For example, in HIV-1 infection the tricyclic compounds can be used in a therapeutic regimen that includes nucleoside analog therapy an protease inhibitor therapy.

Vaccine Formulations Comprising the Tricyclic compounds of the Invention

In another aspect, the invention also provides an isolated and inactivated virus, where the virus is inactivated by a method comprising contacting the virus with a tricyclic compound compound of the invention, wherein contacting said virus with said compound inactivates said virus. In one embodiment the isolated and inactivated virus further comprises a vaccine formulation..

The tricyclic compound-complexed, inactivated viruses of the invention are used in vaccine formulations that are useful for administration to mammals, particularly humans to treat and generate immunity to of a variety of viral diseases, particularly retroviral infections, such as HIV-1. The vaccine formulations can be given single administrations or a series of administrations. When given as a series, inoculations subsequent to the initial administration are given to boost the immune response and are typically referred to as booster inoculations.

The vaccines of the invention contain as an active ingredient an immunogenically effective amount of a tricyclic compound-complexed, inactivated, virus. Useful carriers are well known in the art, and include, *e.g.*, thyroglobulin, albumins such as human serum albumin, tetanus toxoid, polyamino acids such as poly(D-lysine: D-glutamic acid), influenza, hepatitis B virus core protein, hepatitis B virus recombinant vaccine and the like. The vaccines can also contain a physiologically tolerable (acceptable) diluent such as water, phosphate buffered saline, or saline, and further typically include an adjuvant. Adjuvants such as incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum are also advantageously used to boost an immune response.

Uses of Tricyclic compound Inactivated Viruses and Tricyclic compound-Complexed Proteins

In addition to uses as vaccines, tricyclic compound-inactivated viruses and tricyclic compound-complexed viral proteins have a variety of uses. For example, tricyclic compound-complexed viral proteins or tricyclic compound-inactivated viruses can be used as reagents for the detection of corresponding anti-viral antibodies or as crystallization reagents for X-ray crystallization analysis or other ultrastructural studies, *see, e.g.*, Yamashita (1998) *J. Mol. Biol.* 278:609-615; Wu (1998) *Biochemistry* 37:4518-4526.

Kits

In an additional aspect, the present invention provides kits embodying the methods herein. Kits of the invention optionally comprise one or more of the following: (1) a tricyclic component as described herein; (2) instructions for practicing the methods described herein, and/or for using the tricyclic component; (3) one or more assay component; (4) a container for holding tricyclic compounds, assay components, or apparatus components useful for manipulating tricyclic compounds or practicing the methods herein, and, (5) packaging materials.

In a further aspect, the present invention provides for the use of any compound, kit, or kit component herein, for the practice of any method or assay herein, and/or for the use of any apparatus or kit to practice any assay or method herein.

The invention will be described in greater detail by way of specific examples. The following examples are offered for illustrative purposes, and are intended neither to limit or define the invention in any manner.

EXAMPLES

Example 1. Binding of tricyclic compounds to viral nucleocapsid protein.

Example 1a. High-throughput screen.

A high throughput screen was devised to identify reversible antagonists of the NC-p7 oligonucleotide interaction. A PBS based buffer was designed (PBS/10μM zinc chloride/10mM β-mercaptoethanol/0.05% Tween) to select inhibitors that had non-covalent, non-redox and non-chelating activity. The caveat was that assays done in an excess of either reducing agent or zinc would eliminate the possibility of identifying compounds whose inhibition of NC-p7 was through a covalent, redox or chelating based mechanism. A set of ~2000 compounds, the "Diversity Set" was selected for screening that represents a broad range of three dimensional pharmacophores that are found in the NCI's repository of >140,000 compounds.

For screening, NC-p7 was immobilized on a polystyrene 96 well plate and then blocked with 2% BSA. The plates were washed 3 times with PBS/0.05% Tween, and were then incubated with 5nM biotinylated 28mer and 10μM of the compound (in 20% DMSO) for 1 hour. This 28 base oligonucleotide contains 19 bases from the 3' end of U5 and is one used previously (Fisher *et al., J Virol* 72:1902-9, 1998)and was also used in competitive hybridization experiments by Tsuchihashi and Brown (Tsuchihashi, 1994). The plates were washed again with PBS/0.05% Tween and incubated with nutravidin-horse radish peroxidase (40ng/ml) for an hour to detect bound biotinylated 28mer. The plates were washed with PBS/0.05% Tween and developed with Supersignal reagent. Each plate had 8 positives controls (5mM EDTA) and 8 negative controls (20% DMSO alone). The Z factor (a statistical parameter to evaluate quality of high throughput screens) was calculated for each plate to ensure adequate performance through all the plates utilized in the screen. The activity threshold was established as 100% inhibition and 26 positives were identified.

Among the 26 active inhibitors were five having a xanthenyl ring system relating them to fluorescein or gallein (Figure 1). In addition four of the five active compounds were in a "tetra-hydroxy" configuration (Figure 2). The activity of these samples towards NC-p7 was confirmed using BIAcore solution inhibition analysis.

Example 1b. binding assay

This example shows an assay to determine the K_D of a tricyclic compound for the HIV-1 nucleocapsid protein (referred to in the example as p7).

The activity of the active compounds toward NC-p7 was confirmed using a BIAcore® solution inhibition assay (*e.g.*, Christensen *et al.*, *Anal Biochem* 249:153-164, 1997; Fivash *et al.*, *Curr. Opin. Biotechnol.* 9:97-101, 1998; Fisher *et al.*, *J. Virol.* 72:1902-1909, 1998) in which NP-p7 was incubated with increasing amounts of sample and injected over $d(TG)_4$ immobilized on the flow cell surface. Uninhibited NC-p7 was quantified using a NC-p7 standard curve. All of the five samples were confirmed as active in the BiAcore assay. The dissociation constant for binding to NC-p7 was calculated for each sample (NSC's 119889, 119910, 119911, 119913, 119915 with Kd's of 253, 407, 350, 379, 394nM respectively).

Analysis of related compounds. The NCI repository was searched for samples containing this tricyclic ring structure and an additional 63 were obtained. These samples were assayed for their activity towards NC-p7 using BIAcore solution inhibition analysis and dissociation constants were calculated. To preclude the possibility that the samples, were interfering with the immobilized $d(TG)_4$ on the BIAcore surface, the direct binding of some of the samples to NC-p7 was monitored by quenching of the fluorescence Trp^{37} in NC-p7. Dissociation constants for the binding of the sample to NC-p7 were calculated and plotted against those determined by the BIAcore solution inhibition analysis (Figure 3). For the higher affinity binding samples, there was a good correlation between the two Kd's, although this diminished somewhat with lower affinity binders.

Example 2. Anti-viral activity of tricyclic compounds

The following example shows the assessment of anti-viral activity of the compounds described herein.

In addition to the NC-p7 binding assays, cell based anti-HIV screening was performed in CEM-SS cells, using XTT-cytoprotection assay. Compounds were ranked as active (80-100% protection from HIV infection), moderate (50-79% protection) and inactive (0-49% protection). Generally, samples that had lower Kd's, *i.e.*, bound tightly, were found to be active in the cell based assay. The lead sample from this analysis was NSC 119889 (recorded in the NCI chemical database as 2,3,4,5-tetrabromo-6-(3,6-dihydroxy-9H-xanthen-9-yl)benzoic acid) with a Kd of 259nM. However, analysis of this sample by HPLC and mass spectrometry revealed that it was a complex mixture of compounds. Attempts were

made to purify the active material from NSC 119889, but proved unsuccessful due to the complex nature of the sample. Thus, it could not be confirmed that 2,3,4,5-tetrabromo-6-(3,6-dihydroxy-9H-xanthen-9-yl)benzoic acid was the active component in NSC 19889, although it was suspected that a related chemotype was the active material (*see,* below, Example 3, *Binding of synthetic forms of NSC 157412-10 to NC-p7.*)

Thus, in the initial screening of the DTP diversity set, five compounds displayed potent activity in both nucleocapsid binding and antiviral assays, (NSC's 119889, 119910, 119911, 119913, 119915 with Kd's of 253, 407, 350, 379, 394 nM respectively). Inspection of the structures of these five, as recorded in the DTP database, for common structural features revealed those to be a xanthene ring substituted with hydroxyls at the 3' & 6', or 3',4',5',6' positions and a C-C bond to some substituent at the 9' position, (Figure 1). A selected set of authentic compounds possessing these structural features was then obtained by synthesis or by purification from available materials.

Example 3. Characterization of the binding of an exemplary tricyclic ring compound to NC-p7.

Sample NSC 157412 (2,3,4,5-tetrachloro-6-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid; Kd 1850nM) was recorded in the NCI chemistry database as having chlorine residues substituting the bromine residues found in NSC 119889 and two additional hydroxyl groups on the tricyclic ring. As this sample was found to be less heterogeneous than NSC 119889, active material was purified from NSC 157412 ("1a" in Figure 1).

Twenty milligrams of active material was purified from 150mg of NSC 157412 using a C18 HPLC column and confirmed to be 98% pure by LC/MS. The majority of NC-p7 inhibitory activity (59% of the material recovered from the column) was associated with the major peak absorbing at 254nm. The structure of the active fraction was confirmed to be 2,3,4,5-tetrachloro-6-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid by NMR (purified material designated "1b" in Figure 1). The binding of NSC 157412-10 to NC-p7 was analyzed by solution inhibition analysis (Figure 4). In this assay, 50nM of NC-p7 was inhibited with increasing concentrations of 157412-10. Almost complete inhibition was observed with 50nM of NSC 157412-10. Several replicates of this titration were performed with equivalence points ranging from 35-105nM. In a similar experiment, the binding of

highly purified fluorescein (designated as "5" in Figure 1)to NC-p7 was also analyzed and no inhibition was observed at 10μM (Figure 4).

Binding of NSC 157412-10 to NC variants. The binding of NSC 157412-10 to wild type NC-p7, a mutant where all the cysteines in the two zinc fingers were changed to serines (SSHS, Guo *et al., J Virol.* 74:8980-8, 2000.) and a finger swap mutant where the positions of the two fingers were reversed (2:1 swap, Gorelick, *et al., J Virol.* 67:4027-36, 1993.) was monitored by tryptophan fluorescence quenching experiments (Figure 5). NSC 157412-10 bound equally well to the two mutant NC-p7's compared to the wild type (Figure 5). The binding of NSC 157412-10 to NC-p7 as determined by the tryptophan fluorescence quenching experiments was not stoichiometric. Indeed, the affinity was substantially weaker (IC_{50}'s approximately of 3μM) than that measured by the solution affinity assay. Consistent with this, dissociation constants calculated using the tryptophan fluorescence quenching method were always higher than those calculated by the solution inhibition method for many of the samples structurally related to NSC 119889 (Figure 2).

Binding of synthetic forms of NSC 157412-10 to NC-p7. To investigate the specificity of the interaction between NSC 157412-10 and NC-p7 three synthetic variants were made. Number 4436-42-1 (Figure 6) is the synthetic form of NSC 157412-10 (2,3,4,5-tetrachloro-6-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid), 4436-26-1 is the same as NSC 157412 but the bromine residues have been changed to chlorines. Note that number 4436-8-1 is the synthetic form of NSC 119889 (2,3,4,5-tetrabromo-6-(3,6-dihydroxy-9H-xanthen-9-yl)benzoic acid) (Figure 6a). These compounds were assayed for their activity towards NC-p7 by solution inhibition analysis (Figure 6b). Both 4436-26-1 and 4436-42-1 inhibit NC-p7 stoichiometrically; this further confirms the activity in NSC 157412-10 is the same as 4436-42-1, that of 2,3,4,5-tetrachloro-6-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid. These data also suggest that 4436-26-1 is the active material in the crude NSC 119889 sample.

Sample 4436-8-1 had no activity towards NC-p7, 4436-26-1, which differs from 4436-8-1 in that it has two additional hydroxyls at positions 4 and 5 on the tricyclic ring. Fluorescein also lacks these two hydroxyls groups and has no activity towards NC-p7. Thus, these two hydroxyl groups can play a role in the inhibitory activity of this class of compound.

Molecular modeling of 4436-42-1 and 4436-8-1. The results of the docking analysis suggest two candidate docking sites for each zinc finger, located on opposite sides of residues involved in zinc coordination. These sites are referred to as the distal and proximal sites for each zinc finger, and yield four putative docking sites. Among the ligands tested, 4436-8-1 yielded acceptable initial dockings at both the distal and proximal sites of the amino terminal zinc finger and only the distal site for the carboxyl terminal finger. 4436-42-1 could be docked initially at only the proximal sites of each zinc finger. Based on minimal rounds of minimization and molecular dynamics simulations, 4436-42-1, when docked at the proximal site of the carboxyl terminal zinc finger, yields the lowest relative total energy score. In general, the total energy scores for 4436-42-1 at this site were consistently lower than the lowest energy scores for 4436-8-1 when at all candidate sites. As a result of this calculation, the best docked positions of 4436-42-1 were further analyzed. Two orientations were explored, and using their relative total energy scores, one conformation was selected. This bound conformation placed the hydroxyl groups positioned into the nucleocapsid protein, to form an almost pincer grasp of the amide nitrogen of GLY35 at a distance of approximately 3.3Angstroms from each hydroxyl. In this configuration, the ether oxygen is positioned at a distance of 4.7A from GLY:N. The carbonyl oxygen of 4436-42-1 is facing into solvent, no closer than 5A from its nearest neighbor; a result suggesting solvent as its most likely interaction. The hydroxyl at the opposite end of 4436-42-1 is positioned within 3A of the carbonyl oxygen of GLY:40. The oxygens of the carboxylic acid on the phenol ring are situated nearly equidistant (3.8A) from the zeta nitrogen and carbonyl oxygen of LYS33.

Based on these observations, it can be concluded that the docked arrangement of 4436-42-1 is largely electrostatic in nature, involving backbone hydrogen bonding interactions and a single salt bridge with NZ:LYS33. Furthermore, based on the relatively stronger interaction energy calculated for this docking of 4436-42-1 versus all dockings of 4436-8-1, it can also be concluded that a stronger binding affinity would be observed for the former compound.

In this example, the presence of four bromine atoms instead of four chlorine atoms on the benzoic acid substituent at position 9' of tetrahydroxylxantenyl compounds has no effect on NC-p7 binding. However, tetrahalogenated analogs of the compounds that lacked hydroxyls at positions 4' & 5' of the xanthene ring did not bind NC-p7. It should also be noted that fluorescein, which also lacks the two hydroxyls at these positions shows no activity towards NC-p7. These groups are predicted to form a pincer grasp of the amide

nitrogen of Gly35. This residue is important in NC-p7 hydrogen bonding to nucleic acid, thus interaction with Gly35 would decrease the affinity for NC-p7 to nucleic acids. Accordingly, the lack of these hydroxyl groups on the compound designated as "3" in Figure 1, would prevent it from interacting with Gly35 leaving it non-inhibitory.

Example 4. HIV-inhibitory effects of NSC 157412-10 and its analogs in MAGI assay

To determine whether the strong binding of NSC 157412-10 to NC-p7 could correlate to a potent anti-HIV activity in cell based assay, we examined HIV-inhibitory effect of NSC 157412-10 and the synthetic variants, 4436-42-1 and 4436-26-1, in MAGI indicator cells. MAGI assay permits the elucidation of anti-HIV mechanisms specifically at early stages of viral replication prior to viral integration (Kimpton, & Emerman, *J Virol* 66:2232-9, 1992). Cells were treated with increasing concentrations of 157412-10 or 4436-42-1 following infection with HIV-1IIIB (estimated MOI = 0.01). Both NSC 157412-10 and its synthetic version, 4436-42-1, demonstrated a significant inhibition of HIV infection in a dose dependent manner with IC50 (concentration to achieve 50% reduction in the number of HIV-infected target cells compared to positive control) of 27 and 21 μM, respectively (Figure 7-A). Strong anti-HIV activity of the synthetic variants, 4436-42-1 and 4436-26-1, was consistently demonstrated in subsequent experiments, while 4436-8-1 showed significantly reduced activity (IC50, mean ± SD: 23 ± 7, 16 ± 2 and 37 ± 3 for 4436-42-1, 4436-26-1 and 4436-8-1, respectively) (Figure 7-B). These results are in excellent agreement with one another further confirming identify for the purified and synthetic compounds.

Example 5. Antiviral activity of tricyclic compounds *in vivo*

This example demonstrates the assessment of anti-viral activity of the tricyclic compounds *in vivo*. As appreciated by one of ordinary skill in the art, a variety of models can be used to show the *in vivo* activity of the compounds. For example, anti-viral activity can be assessed in a model of HIV-1 infection that uses mice engrafted with human cells expressing $CD4^+$ (*see, e.g.*, http://www.niaid.nih.gov/daids/PDATguide/HIVThera.htm). These animals are therefore susceptible to HIV-1 infection.

In this model (SCID-Hu model), SCID mice are implanted with human fetal thymus and liver tissue. HIV-1-infected mice are treated with a subject tricyclic compound and assessed for a reduction in detectable HIV-1. Antiviral efficacy is demonstrated by a

reduction in virus load (p24 and RNA) and protection of $CD4^+$ cells from virus-induced depletion. The compounds can be evaluated either alone or in combination with other agents.

Pharmacokinetic and toxicology evaluations and studies to determine optimal routes and schedules of administration can also be performed.

As appreciated by those of skill, other animal models, *e.g.*, Rhesus monkeys infected with SIV, can also be used to demonstrate the anti-viral activity of the compounds *in vivo*.

METHODS

Reagents. Oligonucleotides were synthesized with biotin at the 3′ end. Recombinant HIV-1 NC-p7 protein contained 55 amino acids and the sequence was from the MN isolate of HIV-1. Wild type, SSHS NC and finger switch mutants were prepared as previously described (Wu *et al.*, *J Virol* 70:7132-42, 1996; Guo *et al.*, *J Virol.* 74:8980-8, 2000; and Gorelick, *et al.*, *J Virol.* 67:4027-36, 1993). The "Diversity Set" of compounds was provided by the Developmental Therapeutics Program, at the National Cancer Institute .

NC-p7 high-throughput screen. The assay was performed using a Tomtec Quadra robotic system. NC protein was immobilized on Costar (Corning, NY) high-bind polystyrene 96 well plates by incubating 100 µl 250nM NC-p7 in phosphate buffered saline (PBS)/ 10µM zinc chloride/10mM β-mercaptoethanol/0.05% Tween overnight at 4°C. 200µl of 2% BSA in PBS/10µM zinc chloride/10mM β-mercaptoethanol/0.05% Tween was added and incubated at room temperature for 1-2 hours in order to block the plates. The plates were then washed 2 times with 200µl PBS/10µM zinc chloride/10mM β-mercaptoethanol/0.05% Tween using a Titertek M96V plate washer and stored overnight at 4°C. 5nM biotinylated 28 base oligonucleotide (5' GACTTGTGGAAAATCTCTAGCAGTGCAT 3') in PBS/10µM zinc chloride/10mM β-mercaptoethanol/0.05% Tween was added to each well followed by 10µM of test compound (in 20% dimethyl sulphoxide) from the Diversity Set and allowed to incubate at room temperature for 1 hour. Plates were then washed 3 times with 200µl PBS/0.05% Tween. Binding of the biotinylated 28mer was measured by adding 100µl 1:20000 dilution of nutravidin-horse radish peroxidase (stock 0.8mg/ml, from Pierce Chemical Co. Rockford, Il) and incubating at room temperature for 1 hour. The plates were washed 3 times with 200µl PBS/0.05% Tween. Plates were developed by adding 100µl of Supersignal (Pierce Chemical Co. Rockford, Il) and bioluminescence was measured in a

Wallac Victor plate reader. Each plate had eight positive (5mM EDTA in PBS) and eight negative (20% DMSO alone) controls. The hit threshold was set at 100% inhibition.

Anti-HIV activity assays. Anti-HIV activity was initially screened in a human T-cell line CEM-SS, infected with HIV-1_{RF} in the absence or presence of increasing concentrations of the test compound. The protection from HIV-induced cytopathic effect was determined by the 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay, as previously described (Weislow *et al, J Natl Cancer Inst* 81:577-86; 1989). Specifically, cells were plated in 96-well plates at $5x10^3$ cells/well and infected with HIV-1_{RF} (MOI =0.3). Serial dilutions of compounds ($6.36 x10^{-8}$ to $2x10^{-4}$M) were added to the cells in a final volume of 200µl. In each experiment, AZT and dextran sulfate were included as control compounds for anti-HIV activity. The cells were maintained at 37°C with 5% CO_2-containing humidified air for 6 days. Cell viability was quantified by absorbance at 450nm after 4-hour incubation with XTT (0.2mg/ml). Controls were included to correct absorbance readings of naturally colored compounds. Toxicity of the compounds was determined simultaneously on the same plate in uninfected CEM-SS cells.

The multinuclear activation of a galactosidase indicator assay (MAGI assay) was employed to further characterize anti-HIV activity of selected compounds, as previously described (Kimpton & Emerman *J Virol* 66:2232-9,1992) with modifications. Briefly, the HeLa-CD4-LTR-β-gal indicator cells were plated in a 96-well tissue culture plate at 10^4 cells/well 24 hours prior to the assay, and infected with HIV-1 when cells were generally 20 to 30% confluent. The cells were cultured at 37°C in 5% CO_2-containing humidified air in the absence or presence of test compounds for 48hrs, followed by fixation with 1 % formaldehyde and 0.2 % glutaraldehyde in PBS for 5 min. The cells were then washed with PBS and incubated in staining solution, containing 4 mM potassium ferrocyanide, 4 mM potassium ferricyanide, 2 mM $MgCl_2$, and 0.4 mg/ml X-Gal. HIV-infected indicator cells, which appeared as blue cells, were counted under a light microscope.

Surface Plasmon Resonance (SPR) experimental methods. SPR was performed with the Biacore instrument from Pharmacia Biosensor AB (Uppsala, Sweden). SPR experiments were performed essentially as described previously (Fisher *et al.,. J Virol* 72:1902-9, 1998). CM5 sensor chips were modified with biotinylated $d(TG)_4$. The buffer for all SPR experiments was 10mM Hepes pH 7.5, 150mM NaCl, 5% DMSO, 0.04% polyethylene glycol

(PEG) 20000, 1μM $ZnCl_2$, 0.005% Tween20, 100μM Tris[2-carboxyethyl]phosphine (TCEP), 5mM β-mercaptoethanol. Solution affinity experiments were performed as follows; 50nM NC-p7 was incubated with decreasing amounts of compound and incubated at room temperature for 30mins. The solution was then injected over the $d(TG)_4$ immobilized on the sensor chip and the surface regenerated by with two successive pulses of 0.1% sodium dodecyl sulfate (SDS)-3mM EDTA. "Uninhibited" NC-p7 was then quantified from a NC-p7 standard curve. Dissociation constants were calculated by fitting the data with the following equation:

$$= \frac{B - A - K_D}{2} + \sqrt{\frac{1}{4}(A + B + K_D)^2 - AB}$$

Fluorescence methods. Equilibrium binding isotherms for the binding of the small molecules with NC were obtained by monitoring changes the emission intensity of trp^{37} in NC-p7 upon complex formation with the small molecule (Vuilleumier *et al,. Anal Biochem* 244:183-5, 1997). Measurements were made using a Aminco Bowman Series 2 Fluorimeter (Rochester, NY), with excitation at 295nm (2.5nm bandwidth) and emission at 350nm (10nm bandwidth). Increasing amounts of compound were added to 400nM NC-p7 in 10mM Hepes pH 7.5, 150mM NaCl, 5% DMSO, 1μM $ZnCl_2$, 100μM TCEP 0.04% polyethylene glycol 20000 and 5mM β-mercaptoethanol in a quartz cuvette (Hellma Worldwide, Plainview, NY). Corrections were made of intrinsic fluorescence of the buffer and compound. Binding of the compound to NC-p7 results in quenching of the tryptophan fluorescence. Dissociation constants were calculated by fitting the data with the following equation:

$$I = I_0 - (I_0 - I_t)\left(\frac{((N_t + L_t)K_{obs} + 1) - \sqrt{((N_t + L_t)K_{obs} + 1)^2 - 4 N_t L_t {K_{obs}}^2}}{2 L_t K_{obs}}\right)$$

Molecular Modeling: Methods of virtual ligand docking were used to explore possible binding modes for selected ligands. This analysis was conducted in three stages. In the first stage, candidate binding sites on the amino and carboxyl zinc fingers were determined by scanning their surfaces for sterically and energetically favorable ligand binding sites. This analysis used information about local geometry and chemical composition of subregions on the target surface for selecting candidate sites. Relative rankings of the potential interaction sites were based on a scoring scheme derived from a statistical analysis of all known protein-

ligand complexes. It has been shown that application of this method to new crystal complexes indicated that the correct ligand interface was found within the top 5 percent of the best candidate sites (Young *et al., Protein Sci.* 4:1881-1903, 1995). The second stage of the analysis consisted of docking the test ligands at the candidate binding sites. Docking the candidate ligands was based on geometric considerations using a 'geometric hashing technique' which has been found to rapidly determine a family of possible binding geometries for each ligand. Each of the possible binding arrangements was further refined to determine those positions with the maximum binding strength between ligand and target. A previously published model of ligand binding (Wallqvist & Covell, *Proteins* 25:403-419, 1996) was used to select the best binding geometries. This model is based on the atomic preferences of adjacent surfaces buried within a binding interface and has been shown to accurately predict ligand binding strengths and assess the relative contributions of atomic interactions within a binding interface (Covell & Jernigan, *J. Molec. Struct* 423:93-100, 1998). The model has been extended as an adjunct to computational docking and has been useful for identifying ligands effective against NCp7 targets (Turpin *et al., J. Med. Chem* 42:67-86, 1999) and providing testable hypotheses about the modes of action of candidate compounds to inhibit each molecule's function (Huang *et al., J. Med. Chem* 41:1371-1381,1998).

The final stage of docking was obtained from successive *in vacuo* molecular dynamics and energy minimizations using the CVFF91 force field within Discover97.0(MSI) based on the candidate geometries obtained from steps one and two as outlined above. This final step resulted in changes in ligand and target geometries, primarily to eliminate energetically unfavorable van der Waals clashes. These dynamics and minimization steps were done repeatedly to achieve the final geometries. Local exploration of the final geometries that were used for the analysis indicated trapping in a local energy minimum.

Chemical purification procedure. Chemicals were purified using a Waters Millennium 32 HPLC system equipped with a Waters 996 photodiode array detector and a Waters ZMD mass spectrometer. Purification of the starting material was performed on a Waters 5 μM Xterra C18 HPLC column (300mm X 19mm) using a mobile phase gradient of acetonitrile/methanol-20mM aqueous ammonium acetate pH 4.0 with glacial acetic acid. The eluent from 7 chromatographic runs was pooled based on the presence of a strong 503 AMU peak in positive ion mode. The samples were dried by rotary evaporation to remove the organic solvent followed by lyophillization to remove water.

Synthesis of fluorescein and gallein derivatives: Synthesis of the fluorescein and gallein derivatives was done by condensation of the appropriate phthalic anhydride with an excess of resorcinol or pyrogallol by the "hot melt method" (U.S. Patent No. 5,637,733.). Resorcinol (or pyrogallol) was placed in a round bottom flask and heated to 135 oC (or 140°C) to completely melt the crystals. The halogenated phthalic anhydride was added and temperature raised to 200°C for 2 hrs. After cooling to 40°C the crude reaction mixture was precipitated in ice-cold water. The fine suspension was centrifuged giving a brightly colored pellet, which was washed several times by re-suspending in ice-cold water and centrifuging. After the final washing the pellet was freeze-dried to form a finely dispersed powder.

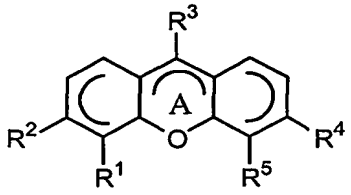
The targeted compound was purified as the fully acetylated derivative (Wei *et al., J. Org. Chem.* 62:6469-6475, 1997). The crude reaction product was dissolved in anhydrous pyridine with a 10 to 20 fold excess of acetic anhydride and magnetically stirred, in an oil bath at 40°C for 8 hours. Excess pyridine and acetic anhydride was removed by partitioning between dichloromethane-aqueous HCl, pH4.0. The organic layer (dichloromethane) was washed with water at neutral pH, dried over anhydrous magnesium sulfate and filtered. The solvent was removed by rotary evaporation. The crude acetylated product was purified by medium pressure liquid chromatography on silica gel, using a step gradient solvent system of dichloromethane-hexane. The enriched fraction was crystallized in methanol-dichloromethane and analyzed by C18 reverse-phase HPLC using a photodiode array detector.

The acetate groups were removed by re-dissolving the crystals in tetrahydrofuran-methanol (1:1) with an excess of ammonium hydroxide (~5 or ~10 equivalents), stirring at room temperature for 8 hours. After this time, if any acetylated product was detectable by C-18 HPLC analysis, a second addition of ammonium hydroxide was added and the reaction was left overnight at room temperature. An equal volume of water was added to the reaction flask and 5% aqueous hydrochloric acid was used to lower the pH to 5. The organic solvents were removed by rotary evaporation, then freeze-dried to yield a finely dispersed color powder. Analysis of the final product by NMR and HPLC-MS-PDA showed the desired product at greater than 95% purity. All compounds were pre-treated with ammonium hydroxide to assure they were tested in the quinoid form.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A method of inhibiting replication of a virus, said method comprising: contacting a nucleocapsid protein of the virus with a compound having the formula:



wherein

R^1 and R^5 are members independently selected from the group consisting of –H, –OH, and =O;

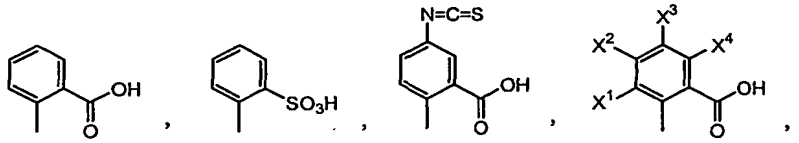
R^2 and R^4 are members independently selected from the group consisting of –H, –OH, and =O;

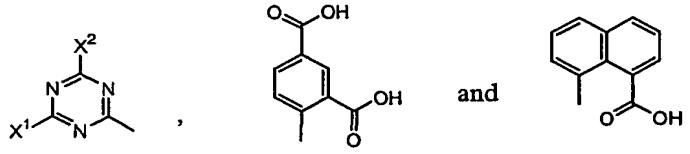
R^3 is a member selected from the group consisting of substituted or unsubstituted alkyl, substituted or unsubstituted alkylene, substituted or unsubstituted cycloalkyl and substituted or unsubstituted aryl; and

ring system A is either saturated or mono-unsaturated.

2. The method according to claim 1, wherein R^3 is aryl substituted with one or more member selected from the group consisting of –COOH, $-SO_3H$, -N=C=S, halogen, and substituted or unsubstituted heteroaryl.

3. The method according to claim 2, wherein R^3 is a member selected from the group consisting of

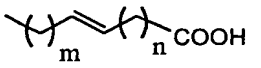


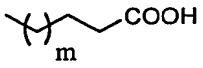


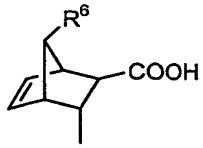
wherein X^1, X^2, X^3, and X^4 are members independently selected from the group consisting of -H, -F, -Cl, -Br, -I and –COOH.

4. The method according to claim 1, wherein R^3 is a member selected from the group consisting alkyl substituted with –COOH, alkylene substituted with –COOH and cycloalkyl substituted with –COOH.

5. The method according to claim 4, wherein R^3 is a member selected from the group consisting of



,


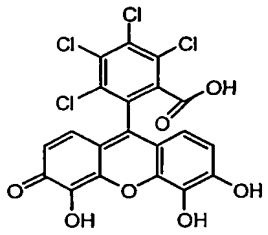
and


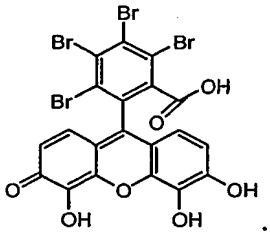
wherein

R^6 is a member selected from substituted alkyl and unsubstituted alkyl; and

m and n are independently integers from 0 to 10.

6. The method according to claim 1, wherein said compound is a member selected from the group consisting of



and


.

7. The method according to claim 1, wherein the virus is a retrovirus.

8. The method of claim 7, wherein said virus is a retrovirus derived from a avian sarcoma and leukosis retroviral group, a mammalian B-type retroviral group, a human T cell leukemia and bovine leukemia retroviral group, a D-type retroviral group, a murine leukemia-related group, or a lentivirus group.

9. The method of claim 8, wherein said retrovirus is an HIV-1, an HIV-2, an SIV, a BIV, an EIAV, a Visna, a CaEV, an HTLV-1, a BLV, an MPMV, an MMTV, an RSV, an MuLV, a FeLV, a BaEV, or an SSV retrovirus.

10. The method according to claim 9, wherein the retrovirus is HIV-1.

11. The method according to claim 1, wherein the contacting step occurs *in vivo*.

12. The method of claim 11, wherein the method further comprises contacting said virus with an anti-viral agent different from the compounds set out in claim 1.

13. The method of claim 12, wherein said anti-viral agent is a anti-retroviral agent that is a nucleotide analogue or a protease inhibitor.

14. The method of claim 13, wherein said anti-retroviral agent is a nucleotide analogue.

15. The method of claim 14, wherein the nucleotides analogue is selected from the group consisting of an AZT, a ddCTP or a DDI analogue.

16. The method of claim 14, wherein the anti-retroviral agent is a protease inhibitor.

17. The method of claim 1, wherein said compound is administered to a human as a pharmaceutical formulation.

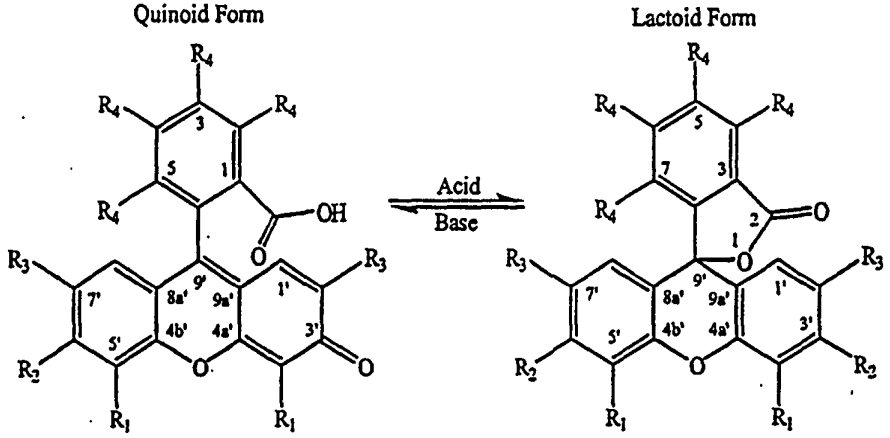
18. The method of claim 17, wherein said compound is administered intra-vaginally or intra-rectally to inhibit the transmission of the virus.

19. The method of claim 17, wherein said compound is administered to an animal as a veterinary pharmaceutical formulation.

20. A pharmaceutical formulation comprising a unit dose of a compound set out in claim 1.

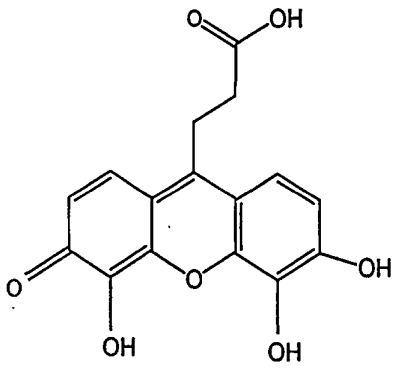
21. The pharmaceutical formulation of claim 20, further comprising a pharmaceutical excipient.

Figure 1. Trivial names and Numbering system for Gallein and fluorescein Derivatives test compounds. The numbering system for the benzoic acid group will change, depending on the form of the molecule (lactoid or quinoid) and the substituents on the ring on the benzoic acid ring system.

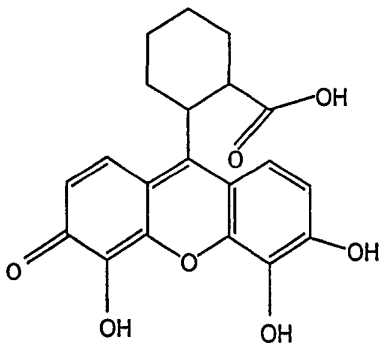


Compound	Lot #	Trivial Name	R_1	R_2	R_3	R_4
1a	NSC157412-J	Tetrachlorgallein	OH	OH	H	Cl
1b	NSC157412-J/10	Tetrachlorgallein	OH	OH	H	Cl
1c	4436-42-1	Tetrachlorgallein	OH	OH	H	Cl
1d	4436-40-1	Tetrachlorgallein Tetraacetate (Lactoid)	OAc	OAc	H	Cl
2	4436-26-1	Tetrabromgallein	OH	OH	H	Br
3	4436-8-1	Tetrabromofluorescein	H	OH	H	Br
4	4436-55-1	Gallein	H	OH	H	H
5		Fluorescein	OH	OH	H	H
6		Eosin Y	Br	OH	Br	H
7		Erythrosine	I	OH	I	H

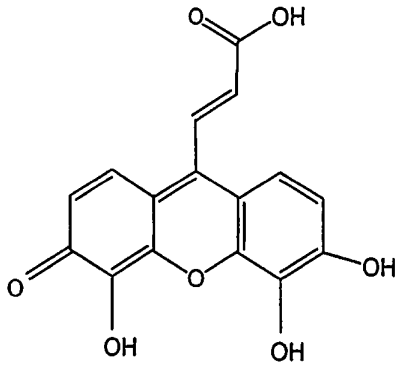
Figure 2: Database structures and NC-p7 binding constants for structurally similar actives from primary screen.



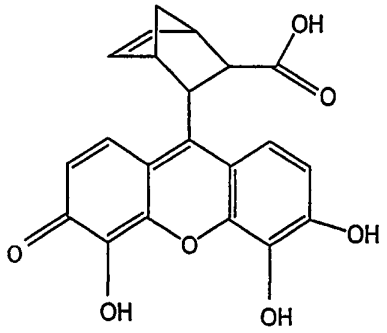
NSC 119915 (394nM)



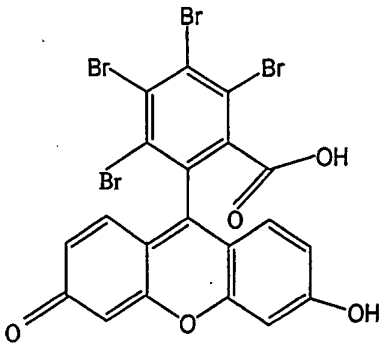
NSC 119910 (407nM)



NSC 119911 (350nM)



NSC 119913 (379 nM)



NSC 119889 (259nM)

Figure 3: Correlation between Kd for compound binding to NC-p7 by solution inhibition or quenching of tryptophan fluorescence.

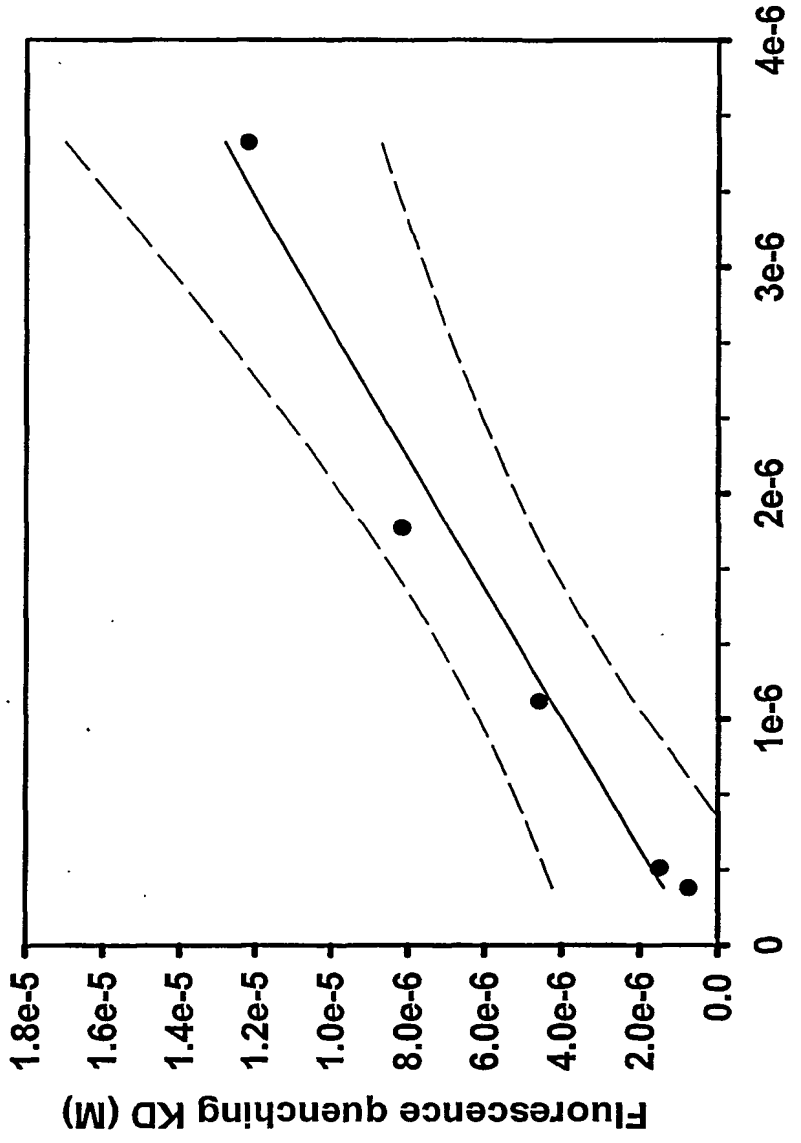


Figure 4: Solution inhibition of NC-p7 with 157412–10 and fluorescein.

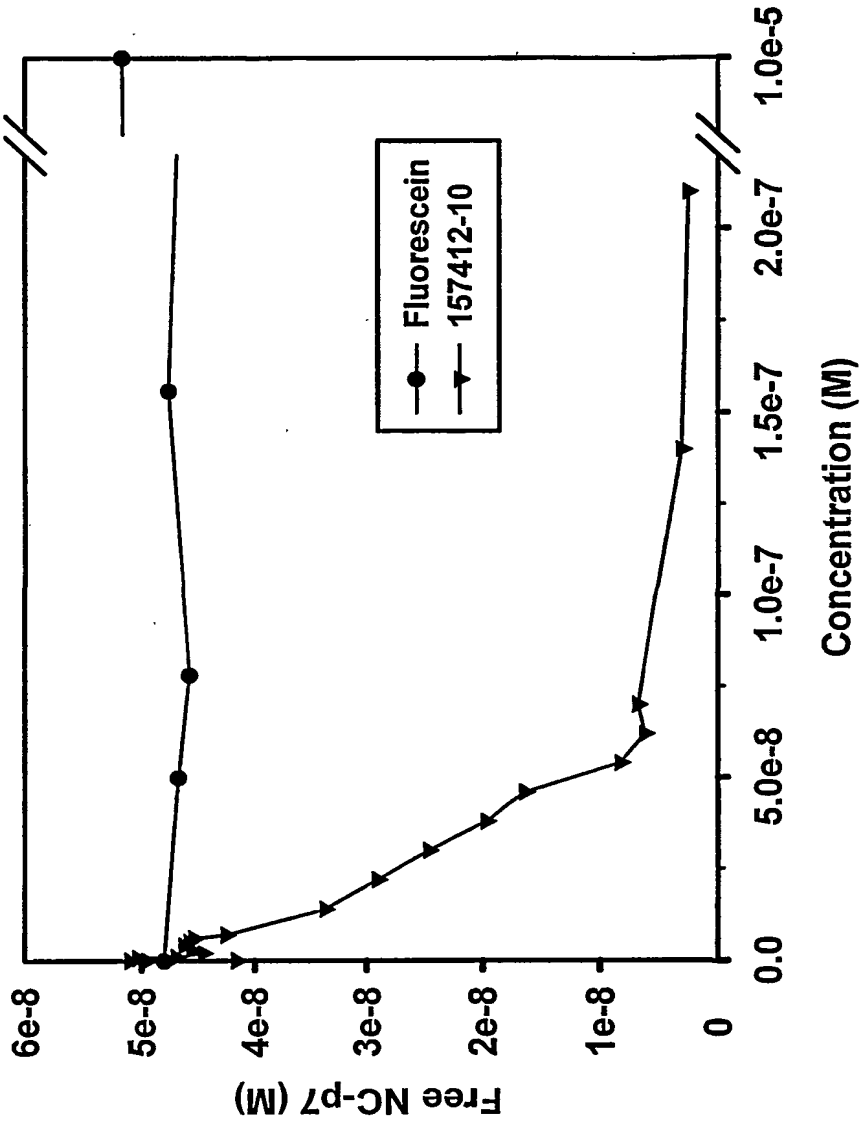
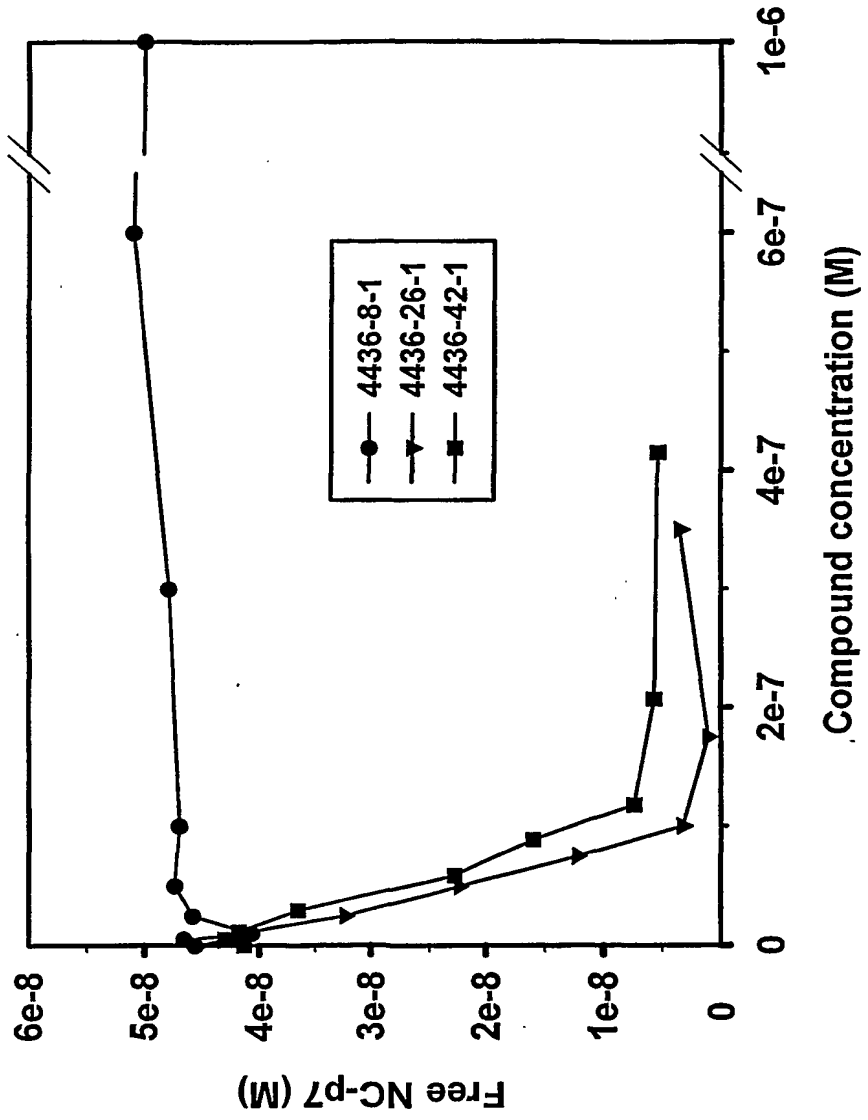


Figure 5: Solution inhibition of NC-p7 with synthetic variants of NSC 157412-10.



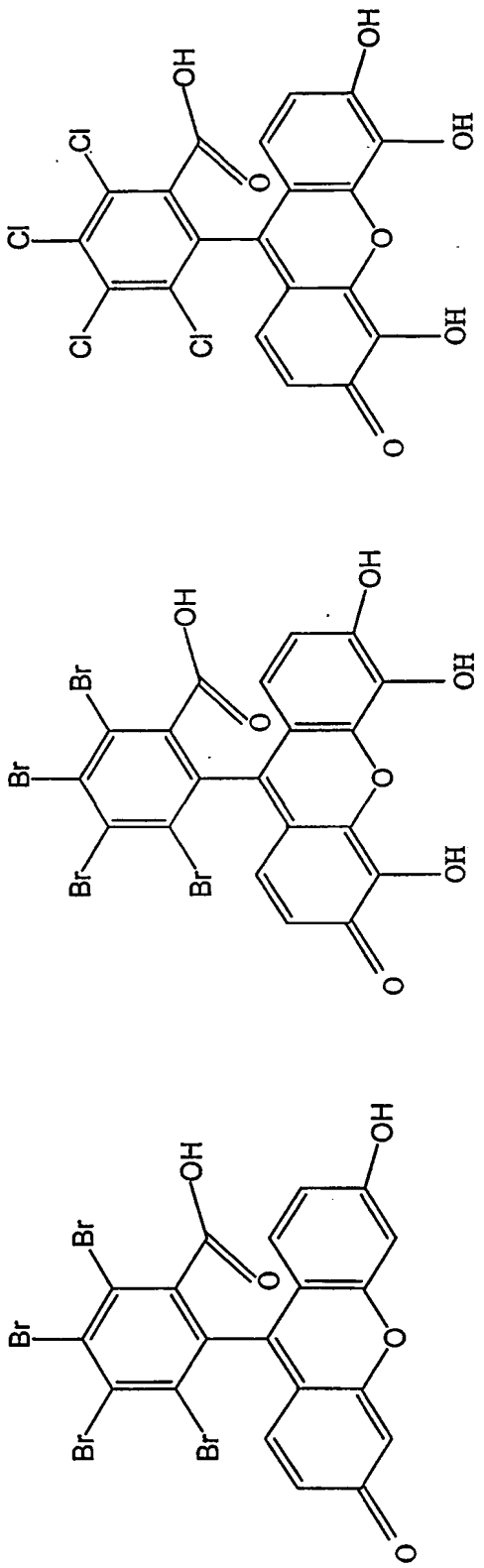
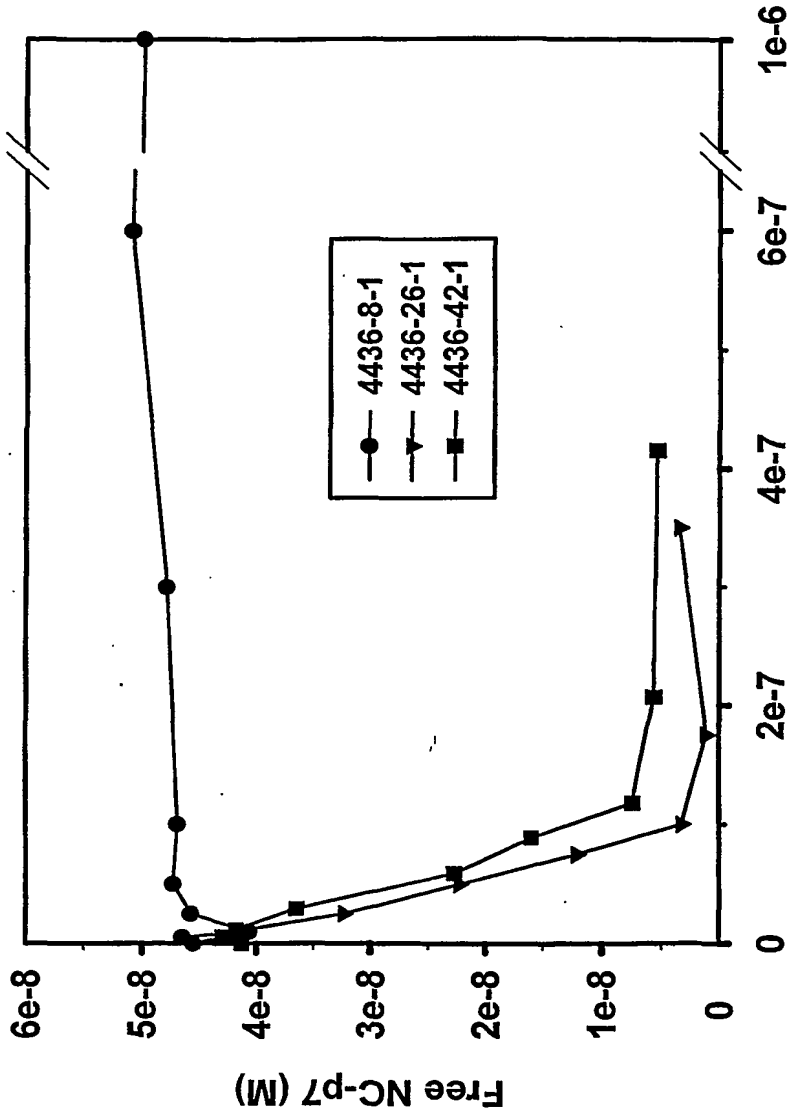


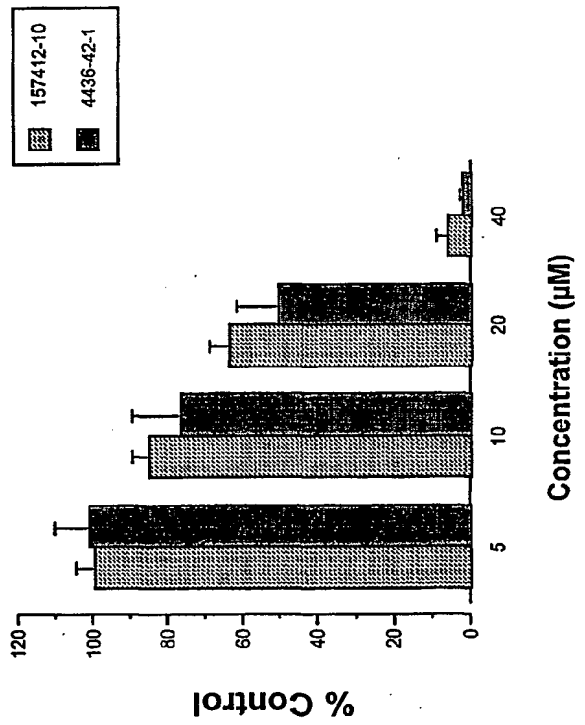
Figure 6a: Structures of synthetic variants of NSC 157412-10.

* Corresponds to structure recorded in NCI chemistry database as NSC 119889.

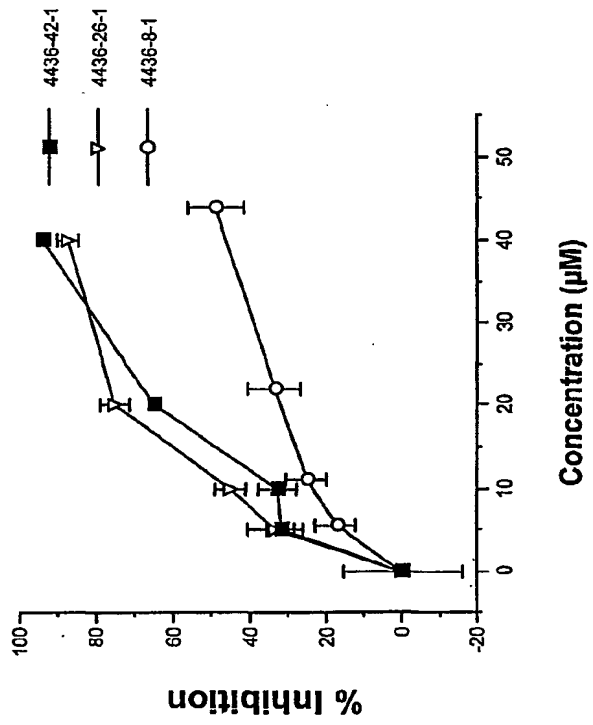
** Corresponds to structure recorded in NCI chemistry database as NSC 157412.

Figure 6b: Solution inhibition of NC-p7 with synthetic variants of NSC 157412-10.




157412-10
4436-42-1
% Control
Concentration (μM)

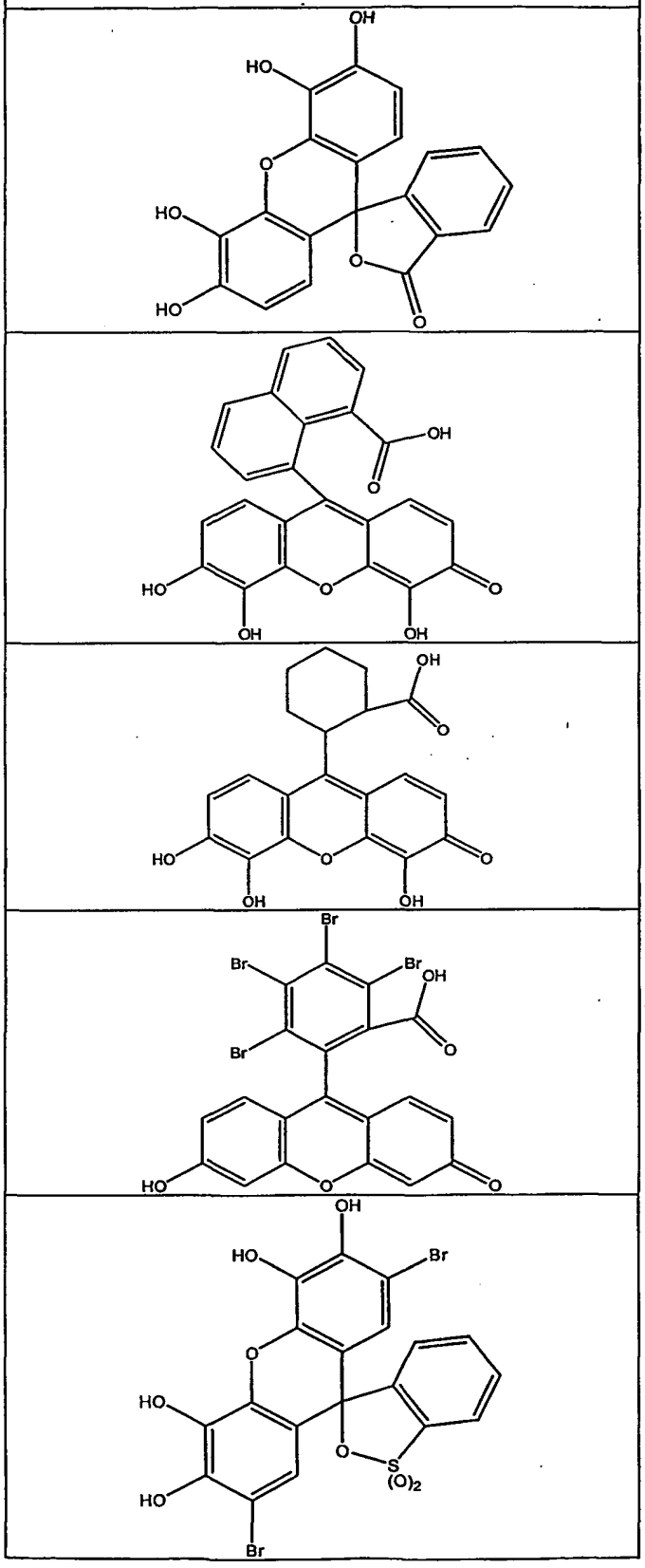
(A)

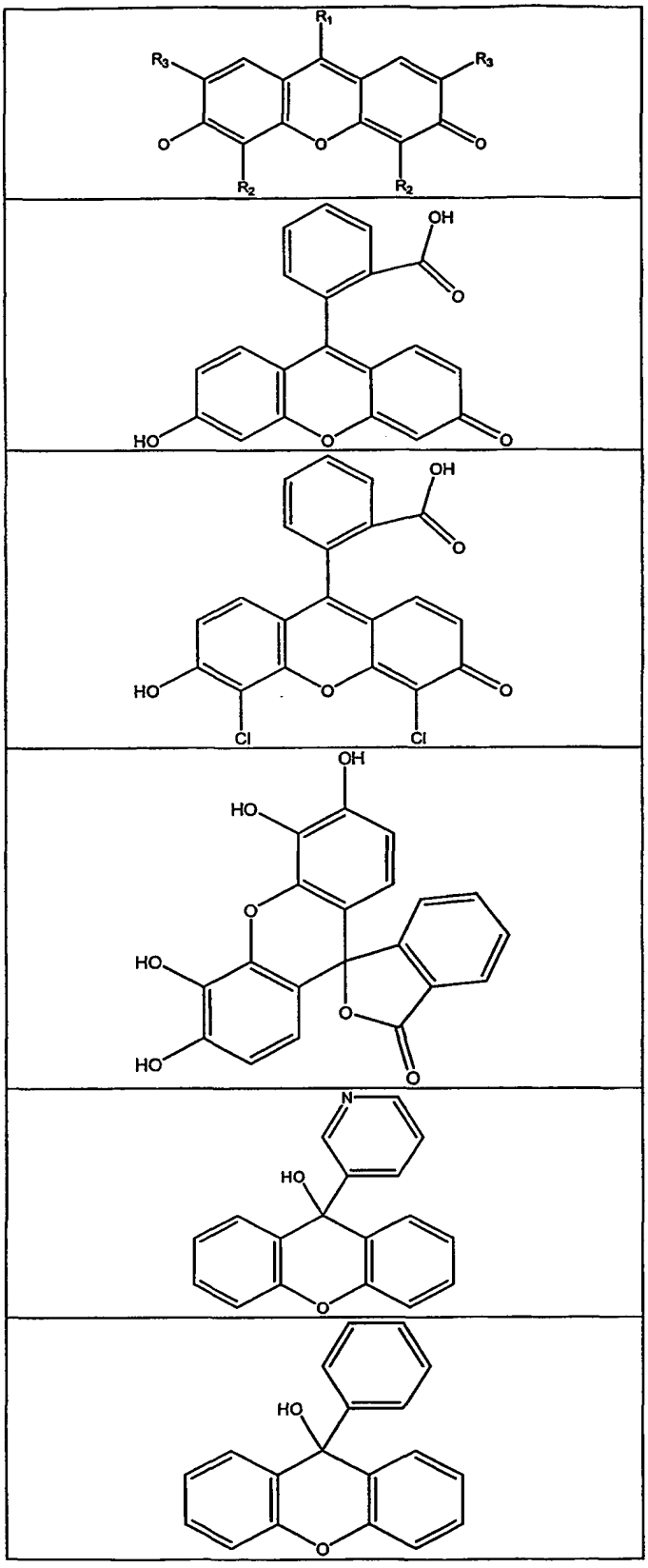

4436-42-1
4436-26-1
4436-8-1
% Inhibition
Concentration (μM)

(B)

Figure 7

Figure 8—Tricyclic Compounds




R1
R3
R3
O
O
O
R2
R2
OH
O
HO
O
O
OH
O
HO
O
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Cl
Cl
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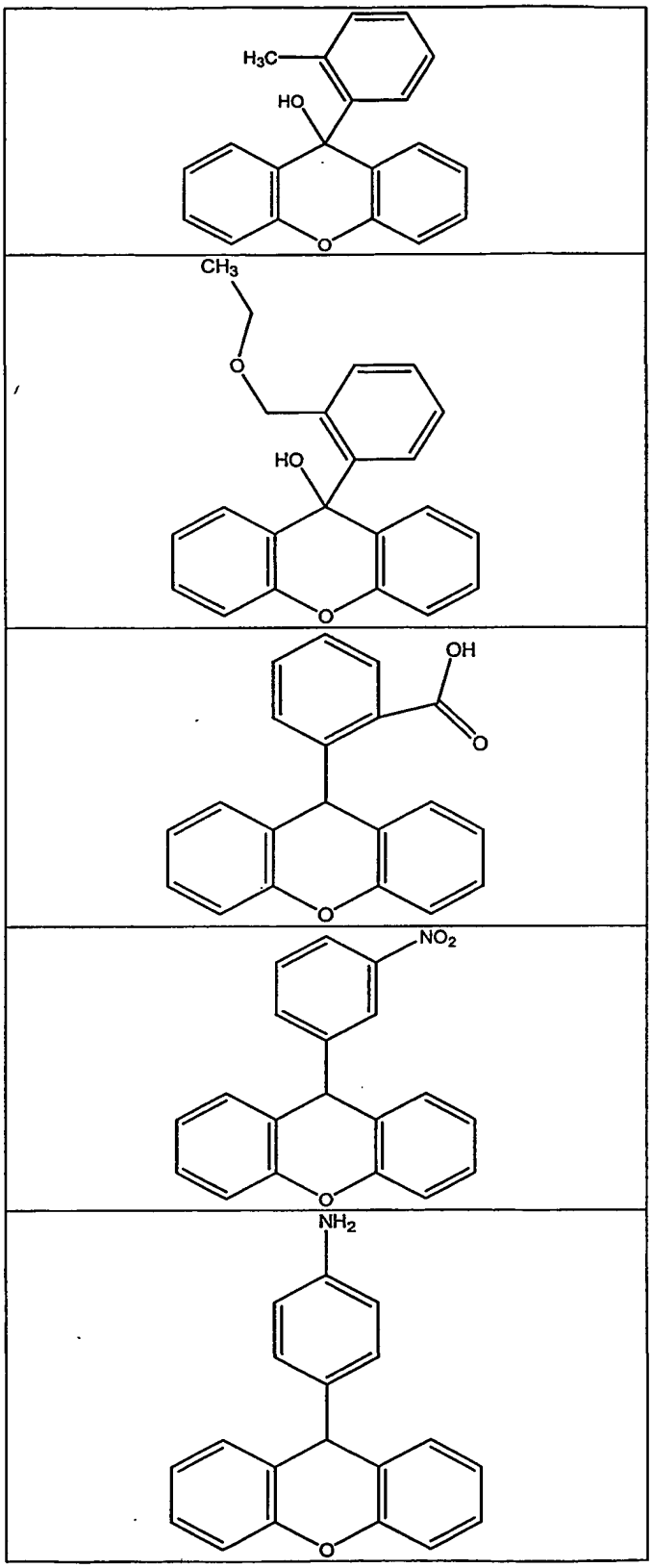
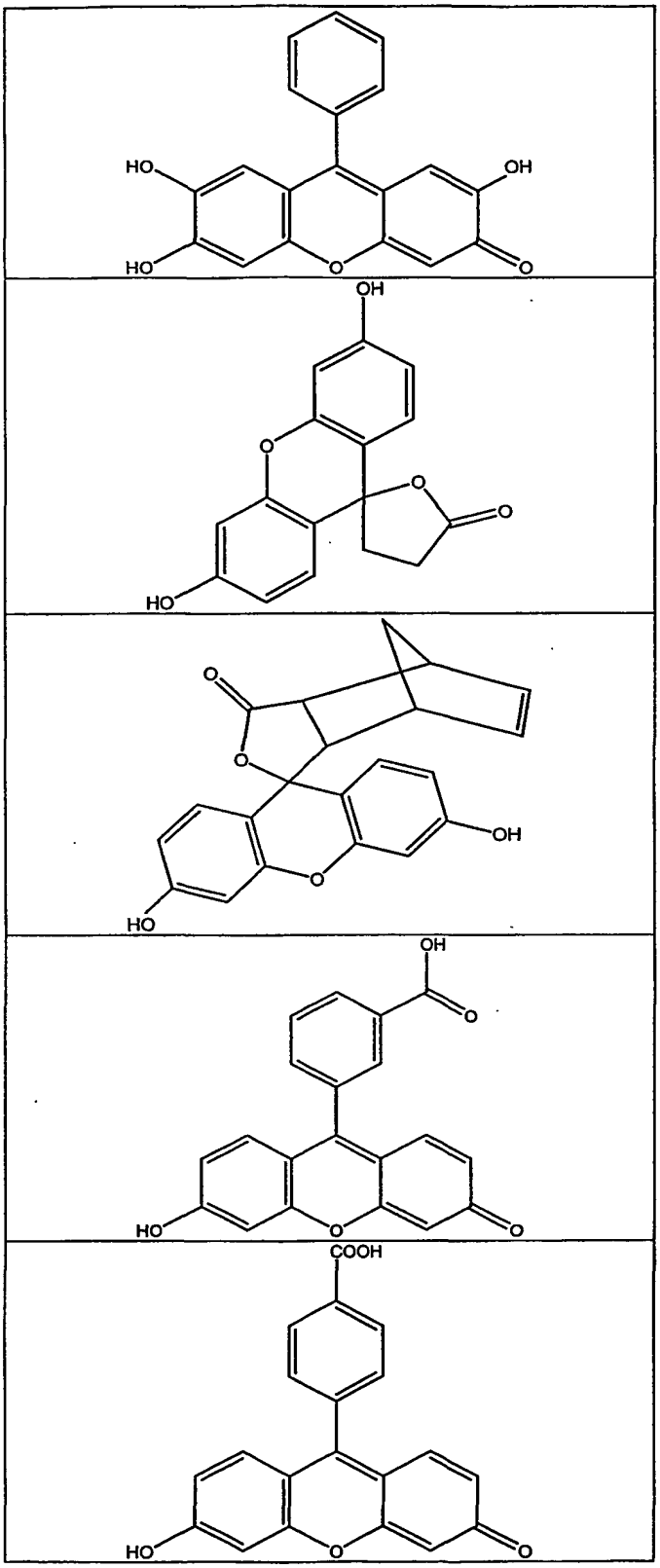
HO
O
HO
O
O
N
HO
O
HO
O


H3C
HO
O
CH3
O
HO
O
OH
O
O
NO2
O
NH2
O


HO
OH
HO
O
O
OH
O
O
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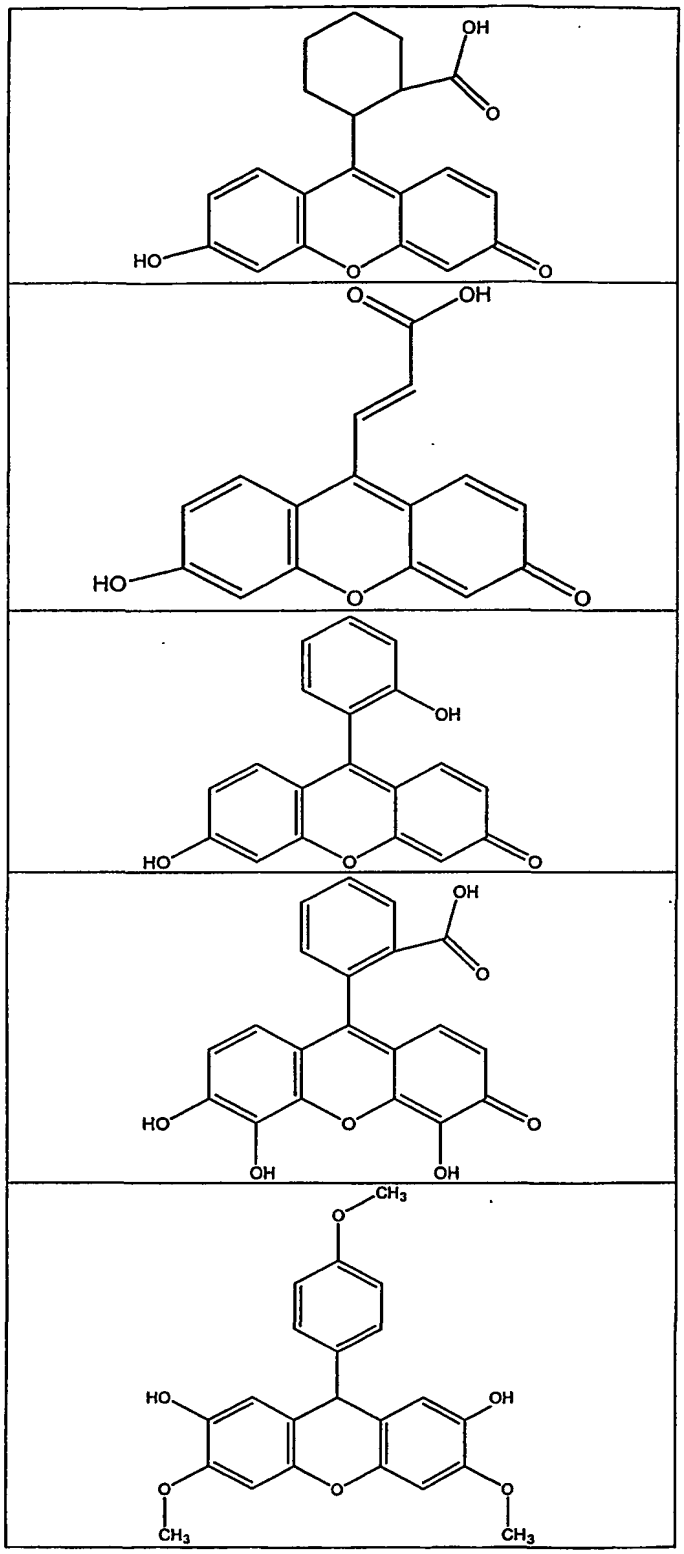
HO
O
O
O
OH
O
HO
OH
O
HO
O
O
COOH
HO
O
O


OH
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HO
O
O
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OH
HO
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OH
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O
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OH
O
HO
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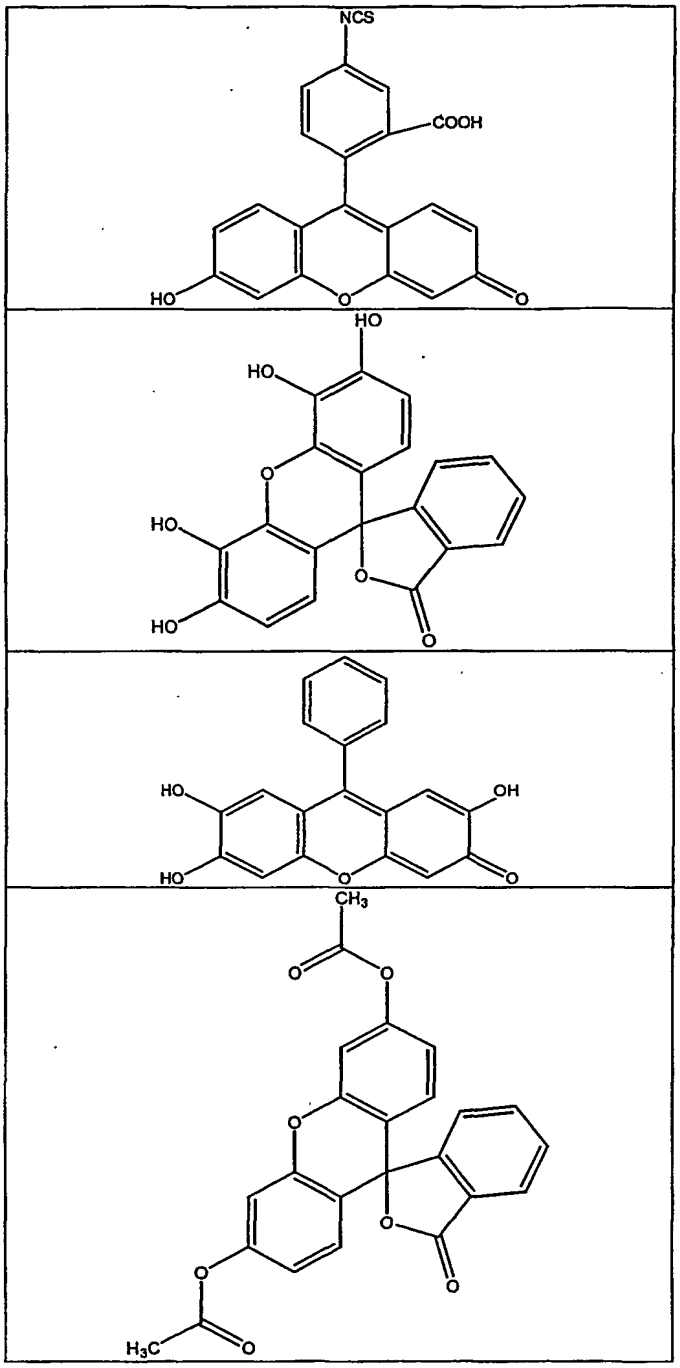
O
OH
OH
CH3
O
HO
OH
O
O
CH3
CH3


NCS
COOH
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HO
HO
HO
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O
HO
O
HO
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OH
O
HO
O
CH3
O
O
O
O
O
O
H3C
O

INTERNATIONAL SEARCH REPORT

Ir ınal Application No
I ... IS 02/03592

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/35 A61K31/53 A61P31/18 C07D311/82 A61K31/352

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 916 668 A (BANYU PHARMA CO LTD) 19 May 1999 (1999-05-19) page 2, line 1,2,9,10 page 2, formula (I) page 16, line 23 - line 29	1,7-21
X	WO 97 40033 A (FUJI IMMUNOPHARMACEUTICALS COR) 30 October 1997 (1997-10-30) abstract page 3, formula pages 11-13, compounds 1-18 page 17, line 23 - line 31 claims 1,2	20,21

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
22 July 2002	29/07/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoepfner, W

INTERNATIONAL SEARCH REPORT

tional application No.
'CT/US 02/03592

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ial Application No
P.. ..3 02/03592

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
EP 0916668	A	19-05-1999	AU	3633997 A	20-02-1998
			EP	0916668 A1	19-05-1999
			US	6140338 A	31-10-2000
			WO	9804554 A1	05-02-1998
WO 9740033	A	30-10-1997	AU	2678997 A	12-11-1997
			WO	9740033 A1	30-10-1997
			US	5965605 A	12-10-1999